Studies on the conservation of sweet potato (Ipomoea batatas (L.) Lam) germplasm

De Goes, Marisa

Award date:
1993

Awarding institution:
University of Bath

STUDIES ON THE CONSERVATION OF SWEET POTATO (*Ipomoea batatas* (L.) LAM) GERMPLASM

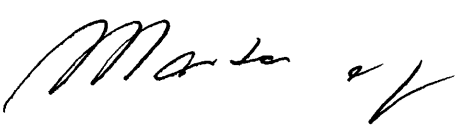
Submitted by

Marisa de Goes

for the degree of Doctor of Philosophy

of the University of Bath, UK

1993



Marisa de Goes

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

I am acknowledged to my supervisor Professor Graham G. Henshaw for his kind guidance during my studies at Bath University.

I also acknowledge Dr. John Beeching for his supervision on the electrophoresis studies.

I am thankful to Mr. Chris Davey and Mr. Peter Clark for their technical support. I also thank Dr. Dermot C. O'Flynn M.B.B.cH.B.A.O. for his kind assistance with the language. I am indebted to all my friends, specially Dr. Florence M. Wambugu, Dr. Enny Sudarmonowati, Dr. Nelum Deshaprya, Mrs. Rosane S. Resende, Dr. Richard I. Samuel, Dr. Denis V. Coury, Ms. Gercina F. Amadeu, my mother and my children for their dedication and psychological support.

Finally my gratitude to Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA) for their support through my scientific career and specially during my PhD studies.

DEDICATION

I dedicate this thesis to the memory of my father Raul de Goes.

ABSTRACT

New procedures for medium and long term preservation of *Ipomoea batatas* germplasm were investigated.

The first involves the use of liquid paraffin (LP) and liquid silicone (LS) overlay inducing slow growth of nodal segments of Papota, TIB-10 and CN-1367-2 genotypes. The plantlets under both oil treatments were very short with extremely reduced leaves and many axillary buds; the newly developed shoots also developed axillary buds. Explants treated with LP were more affected by this shoot proliferation than others treated with LS. Papota showed up to 75% and 58.3% plantlets survivors after 12 month under LP and LS, respectively but no survival at 17 months; TIB-10 showed 58.3% and 16.7% survival and CN-1367-2 showed 41.7% and 50% survival after 17 months under LP and LS, respectively. The recovery of plantlets after the storage period was successful and LP was considered to be the most satisfactory treatment.

The second procedure was based on the cryopreservation of root-tips obtained from *in vitro* cultured plantlets of Papota, TIB-10 and Brondal, using 10% dimethyl sulfoxide (v/v) as a cryoprotectant. Explants slowly frozen at $0.5^{0}C.min^{-1}$ from $5^{0}C$ to $-20^{0}C$ and allowed to stabilize at this temperature for 10 minutes before being plunged into LN ($-196^{0}C$) showed up to 25% survival on the basis of the fluorescein diacetate viability assay.

Additional research involving electrophoresis and *in vitro* root culture complemented the conservation studies. The results showed that an esterase isozyme electrophoresis procedure using roots grown *in vitro* could provide a method for the rapid identification of duplicates in sweet potato germplasm collections. Direct regeneration of shoots from roots growing on basal medium MII-m without growth regulators or containing a low level of NAA could provide the basis for plant recovery from cryopreserved root-tips.

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

ABBREVIATIONS

AcPh	acid phosfatase
BA	benzylaminopurine
cal	calories
CIAT	International Centre for Tropical Agriculture
CIP	Internatinal Potato Centre
cm	centimetre(s)
DMSO	dimetyl sulfoxide
EST	esterase
EDTA	ethylenediaminetetra-acetate
FDA	fluorescein diacetate
g	gram(s)
$g.l^{-1}$	grams *per* litre
GA_3	gibberellic acid
GOT	glutamic-oxaloacetic transaminase
ha	hectare
h	hour
IAA	indole-3-acetic acid
IBPGR	International Board for Plant Genetic Resources
IVAGs	*in vitro* active genebanks
IVBGs	*in vitro* base genebanks
Kg	kilogram(s)
$Kg.ha^{-1}$	kilograms *per* hectare
LAP	leucine amino peptidase
LN	liquid nitrogen
LP	liquid paraffin

LS	liquid silicone
m	metre(s)
M	molarity
mA	milliamps
mg	milligram(s)
$mg.l^{-1}$	milligrams *per* litre
ml	millilitre(s)
m	millimetre(s)
MS	Murashige and Skoog (1962) nutrient medium
MII-m	nutrient medium for initiation of sweet potato meristems (see table 1.2)
MII-t	nutrient medium for transference of sweet potato plantlets and nodal segments (see table 1.2)
MII-p	nutrient medium for storage of sweet potato stocks (see table 1.2)
NAA	1-naphthaleneacetic acid
PER	peroxidase
PVP-40	polyvinil pirrolidone 40.000M
Rf	relative electrophoretic mobility
TEMED	N,N,N',N'-tetramethylethylenediamine
$ton.ha^{-1}$	tonnes per hectare
V	volts
v/w	volume/weight rate
w/v	weight/ volume rate
uM	micromoles

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Origin and Dispersal of Sweet Potato

Sweet potato (*Ipomoea batatas* (L.) Lam.) is one of the two species of the family Convolvulaceae which are cultivated commercially for human consumption in tropical and subtropical areas of the world. There are about 500 wild species of *Ipomoea*, many of these have local value as food, medicines and religious rituals (see reviews by Onwueme, 1978; Villareal *et. al.*, 1979; Martin, 1984 and Huaman and De la Puente, 1988).

Sweet potato is not known in its original wild form. The evolutionary steps by which the crop originated remain uncertain and controversial, however there is an agreement that *I. batatas* is a natural hybrid closely related to *I. trifida* (Jones, 1967; Nishyiama, 1971, 1982; Nishyiama *et al.*, 1975; Austin, 1977, 1978, 1988). Recently, Austin (1988) suggested *I. triloba* is the second candidate as a close relative to sweet potato. However all researchers agree that more studies should be done in order to clarify the origin of *I. batatas*.

Sweet potato was one of the first plants to be domesticated (Simpson and Orgonzaly, 1986; Austin, 1988). The exact location of its origin is uncertain but links can be found in the theory of a pre-Colombian contact between the Old and the New World. Evidences suggest that

the crop was cultivated by natives in both America and South Pacific islands before the existence of European contact. Nevertheless, there is a wider range of genetic diversity in populations of Northwestern South America and parts of Central America than Southeast Asia and Oceania. Also evidences from linguistic and archaeological sources provide the strongest support for that of an American origin (see reviews by Groth, 1911; O'Brien, 1972; Yen, 1982; Simpson and Orgonzaly, 1986 and Austin, 1988).

Presently, despite the absence of concrete proof, there exists a general belief that sweet potato was carried from South America across the sea to Polynesia, probably somewhere in Samoa, and then distributed to other South Pacific islands (O'Brien, 1972; Yen, 1982; Simpson and Orgonzaly, 1986; Austin, 1988). Northwestern South America is probably the primary centre of diversity and secondary centres occur in Southern Peru and Central America. Other secondary centres outside of the Americas are in China, Southeast Asia, New Guinea and East Africa (Yen, 1970; Austin, 1978, 1988; Jones *et al.*, 1986; Huaman and De la Puente, 1988).

Columbus'es introduction of the crop to Europe has been well documented. There is also ample evidence which shows that the Portuguese explorers introduced it into Africa, Asia and India in the sixteenth century (Groth, 1911; Baker, 1970; O'Brien, 1972; Yen, 1982).

1.2 Agronomic Characteristics

1.2.1 The plant

Sweet potato is a herbaceous vine plant which produces starchy edible storage roots.

Because of its long cultivation, wide dispersal as food and tendency to spontaneous mutation and hybridization sweet potato is extremely variable (Austin, 1978; Jones *et al.*, 1986). Basically the tuberous roots have six shapes and three flesh colour groups, however there is much greater variability in the genotypes which can have different morphological and physiological characteristics i.e. the growth habit, shape and pigmentation of leaves, roots and flowers, latex production, pest and disease reaction (Huaman, 1988).

Sweet potato is normally cultivated as an annual crop, however, in natural circumstances it is a perennial plant. Only local farmers of some less developed regions where sweet potato is part of the subsistence, treat the plant as a perennial, harvesting one or two of the biggest roots each time without killing the plant (Norman *et al.*, 1984; Jones *et al.*, 1986; Collins, 1988).

1.2.2 Propagation

The vegetative propagation of sweet potato is from vine cuttings, although it may also be propagated from storage roots and leaves. Vine cuttings of up to 30 cm are obtained from plants of the previous crop or from storage roots, which are able to produce many sprouts.

Leaves with their petioles can be easily rooted and have been used as phytomodels in research (Wilson, 1973).

Seed propagation is only used for breeding (Norman *et al.* 1984).

1.2.3 Climate

Sweet potato adapts to a wide variety of climatic conditions. It can be cultivated from 40^0N to 32^0S and from sea level to 3,000 m above sea level. It grows best where the average temperature does not fall below 24^0C. The plant is very sensitive to frost, requiring a minimum frost free period of 4-8 months, and temperatures below 10^0C can damage the crop (Martin, 1984).

Sweet potato is quite resistant to drought, however the yields can be seriously reduced if severe water stress occurs during the period of root storage (Kassan, 1976).

1.2.4 Diseases

Sweet potato is susceptible to diseases caused by bacteria, fungi, nematodes, viruses, mycoplasmas and abiotic factors. The most important diseases are caused by root pathogens or pathogens which are capable of spreading systemically through the plant. By the time signs appear it is often too late to restore the plant to its previous state. The process of vegetative propagation itself helps to perpetuate the pathogenic survival in the production cycle. Therefore, infection control is best approached by prevention rather than treatment (see review by Clark and Moyer, 1988).

1.2.5 Harvest and storage

The majority of the world's sweet potato are harvested by manual digging, once harvested they are also sorted manually. Even in more developed regions, harvesting has not always been mechanised. Mechanisation with disc ploughs or chain diggers removes the root from the soil but collection and sorting is still done by hand (Onwueme, 1978; Wilson and Abrams, 1982).

After the harvest the roots are cured at 27-29.5^{0}C with a high relative humidity (85-90%). In the tropics this process occurs naturally. Curing is necessary in order to minimize infections by microorganisms and increase the resistance to damage during handling (Onwueme, 1978).

Storage is recommended at 13-16°C with 85-90% relative humidity. Most of the sweet potato produced in the tropics is not stored under controlled conditions consequently; post harvest loses of 25% are normal (Onwueme, 1978; Euroconsult, 1989).

1.2.6 Breeding

From a breeding point of view, sweet potato is a very difficult crop. Flowering is scarce, mainly in sub-tropical regions, and seed production is very low whatever the region. Sterility is very common and most of the cultivars are self-incompatible highly heterozygous and variable (Edmond and Ammerman, 1971; Broertjer and van Harten, 1978; Martin, 1982, 1984; Jones *et al.*, 1986). It has been also reported that spontaneous mutation occurs quite frequently (Clark and Moyer, 1988).

The low yield in many of the developing countries (see section 1.3.1) occurs mainly because of the deficiency of specific cultivars for each region (Collins, 1988; Miranda *et al.*, 1988).

1.3 World Importance

1.3.1 World production

The crop is grown in more than a hundred countries in the tropical, sub-tropical and warm temperate regions of the world. It is the seventh in the world's top crops

and the third among the root/tuber crops after potato and cassava (Table 1.1) (FAO, 1990).

Table 1.1 World's tuber/root production (1987/89 average) (FAO, 1990).

CROP	PRODUCTION m.m.ton	%	YIELD ton.ha^{-1}
Potato	277	47	15
Cassava	142	24	8
Sweet potato	131	22	14
Yams	24	4	9
Taro	6	1	5
Others	3	2	-
Total	582	100	-

Legend: m.m.ton.= million of metric tonnes

Accurate data from sweet potato production is very difficult to obtain mainly because it is often a subsistence crop in the developing countries (Onwueme, 1978; Collins, 1988).

The exportation of sweet potato is insignificant, and exporting countries include Japan, Egypt and Israel (Euroconsult, 1989). Developing countries grow and consume 98% of the world production; China alone is responsible for 80%, Africa for 5% and Latin America for 2% (Horton, 1988; Huaman and De la Puente, 1988). Japan and United States are the only industrial countries which grow significant amounts of the crop (Euroconsult, 1989).

1.3.2 Nutritional value

Sweet potato is an important source of carbohydrate, it has a high caloric value and it can provide about 113 calories *per* 100 g, whereas potato provides only 75 calories, although despite the caloric difference potato may elevate blood sugar levels more than sweet potato (Martin, 1984).

Both roots and leaves are important sources of vitamins, especially ascorbic acid. The dessert cultivars contain 121% of the recommended dietary supply allowance per day of vitamin A (Clark and Moyer, 1988). The leaves are richer than roots in protein, fibre, calcium, iron, phosphorus and potassium, but are poorer in carbohydrates and have less vitamin A than the dessert type roots (Martin, 1984).

Table 1.2 Nutritional composition of sweet potato in 100 mg (Martin, 1984).

	ROOTS	LEAVES
Calories	108.00-121.00	42.00
Moisture (%)	73.30-68.80	86.70
Protein (g)	1.00-1.90	3.20
Fat (g)	0.30-0.20	0.70
Carbohydrate (g)	25.60-28.50	8.00
Fibre (g)	0.80-1.00	1.60
Ash (g)	0.70-1.00	1.40
Calcium (mg)	21.00-33.00	86.00
Phosphorus (mg)	38.00-50.00	81.00
Iron (mg)	0.90-2.00	4.50
Sodium (mg)	31.00	5.00
Potassium (mg)	210.00	562.00
B-carotene eq.(mg)	35.00-2400.00	2215.00
Thiamin (mg)	0.09-0.14	0.11
Riboflavin (mg)	0.04-0.05	0.22
Niacin (mg)	0.70	0.70
Ascorbic acid (mg)	21.00-37.00	17.00

1.3.3 Uses of sweet potato

The main use of sweet potato is in human consumption, where it can be used as a staple, supplementary food or dessert.

The raw storage roots contain a trypsin inhibitor which is easily eliminated by heating (Bradbury *et al.*, 1985). They can be eaten boiled, fried, roasted and baked or can also be processed as wine, chips, flour or starch (Onwueme, 1978; Martin, 1984). In Japan, over half of the annual production is used for wine, starch and alcohol (Simpson and Orgonzaly, 1986).

Fresh leaves and shoot tips are eaten fried, boiled or in soups and stews (Martin, 1984; Villareal *et al.*, 1979). The leaves and shoot tips are easily available throughout the year in the markets of Philippines, Malaysia and Indonesia (Villareal *et al.*, 1979).

Animals can eat the whole plants, fresh or in silage. Vines can be an important source of nutrients for animals, especially in the dry season when sweet potato is more resistant than other forages. There are special cultivars that can be used for forage which produce large amounts of leaves (Onwueme, 1978; Fouda, 1988; Horton *et. al.*, 1989).

Fast spreading genotypes can result in complete ground cover in less than 35 days, as the root system of sweet potato is extensive. Genotypes which spread rapidly can be used in continuous cultivation on steep slopes to prevent erosion (Acland, 1971; Norman *et al.*, 1984).

Medical researchers have pointed out that sweet potato leaves possess unique properties as diuretics and saliuretics (Villareal *et al.*, 1979).

Sweet potato is also used as a house plant in some tropical countries (Huaman and De la Puente, 1988).

1.4 Sweet Potato Germplasm

1.4.1 Genetic erosion

The genetic erosion of sweet potato started in the 17th century at the primary centre of diversity, when the South American natives influenced by the European settlers, changed from the starchy to the sweeter genotypes which were better suited to the European taste (Huaman and De la Puente, 1988). Today it is not known how much useful variability has been lost since then. The erosion expanded around the tropics where over the years local growers selected at first their seed stocks from small unmarketable roots and later trying to repair their mistake, selected part of the highest producing plants to improve yields using generally a narrow genetic basis (Edmond and Ammerman, 1971; Yen, 1982).

1.4.2 Germplasm conservation

The germplasm conservation is related to the activities of collection, disease indexation, quarantine, multiplication, characterization and evaluation, storage and distribution of germplasm (IBPGR, 1986).

In the last decades attempts have been made to develop methods which can reduce or stop the growth of vegetatively propagated plants in order to maintain the crop's gene pools available for future research and breeding under control in laboratories.

Undoubtedly, despite being a method which is still under research, cryopreservation is the ideal system to stop growth and maintain the genetic integrity of plant germplasm in the *in vitro* base genebanks (IVBGs). Even the conservation of active collections does not yet have a well defined technique which can be indicated as a general method to maintain all the vegetative propagated plant germplasm, instead there is a variety of methods which can reduce the growth of groups of genotypes (IBPGR, 1986; Withers, 1984).

Sweet potato is one of the six crops considered by the International Board for Plant Genetic Resources (IBPGR) at the most urgent need for collection, evaluation and conservation regarding their world-wide importance and risks of even more genetic erosion (see review by Ford-Lloyd and Jackson, 1986).

As sweet potato produces orthodox seeds which can be stored at low temperatures (Jones and Dukes, 1982),

the IBPGR recommends the conservation of seeds to maintain the genetic diversity, however the conservation of clones is usually in their vegetative form (Williams and van Stolen, 1988).

To date a limited number of reports were published describing methods which have been tested in the search for the conservation of sweet potato germplasm. Allan (1979) conserved nodal cuttings at reduced temperatures varying from 6 to 28°C, the best results were obtained at 22°C where plantlets were still alive after 55 weeks (about 13 months). Frison and Ng (1981) used 30 $mg.l^{-1}$ mannitol added to the multiplication medium in order to maintain virus-free plantlets. Desanero and Rhodes (1989) obtained minimal growth inducing osmotic stress in the cultures by adding mannitol (30-40 $g.l^{-1}$) to the medium; the interval between sub-cultures increased to 8 and 12 months with 97 and 62% of survival for the 2 tested genotypes, respectively. Recently, Jarret and Gawel (1991) added 0.01 to 10 $mg.l^{-1}$ abscisic acid (ABA) to the basic MS medium and maintained the cultures at 28°C; the results showed complete inhibition of growth with 95% of viability after 90, 180 and 365 days of culture under 1 to 10 $mg.l^{-1}$.

Characterization is obviously one of the most important aspects of germplasm conservation; nowadays beside the field characterization (which is extremely important from the breeding point of view), sophisticated methods based on the fragmentation of DNA molecules by restriction enzyme (RFLPs) methodologies have been

developed in order to give the precise identity of each genotype. However methods such as electrophoresis of isozymes are still a fast and practical tool to be used in the germplasm characterization, especially to identify duplicates. It is estimated that germplasm in general collections of plant germplasm now aggregate several millions due to the extensive number of duplications (Williams, 1989). The maintenance of such duplicates is expensive and could be avoided through the characterization of the genotypes before they are incorporated to the collections.

Electrophoresis of esterase isozymes is the method which has been successfully used for the characterization of the cassava germplasm collection at the International Centre of Tropical Agriculture, in Colombia (CIAT, 1986). Another practical example of the use of isozymes electrophoresis in germplasm conservation is to identify mislabelled material.

1.5 Research Objectives

The main purpose of this thesis was to develop effective techniques for the long and medium term storage of sweet potato germplasm and also techniques which could be helpful for the improvement of the conservation of vegetatively propagated plants.

CHAPTER 2

MATERIAL AND METHODS

2.1 Plant Material

The *Ipomoea batatas* genotypes used for the experiments were originally provided by Dr A. Brunt from the Institute of Horticultural Research (IHR), Littlehampton, in December 1987. The following seven genotypes were used:

1- Brondal
2- CN-1367-2
3- Jersey Orange
4- TIB-9
5- TIB-10
6- Papota
7- Rose Centennial

2.1.1 Growth and maintenance of *in vivo* plants

On arrival from IHR, the disease-free herbaceous vines of sweet potato were immediately potted after their arrival with Fisons C2 Compost and 5 $g.l^{-1}$ N,P,K fertilizer (Fisons-140). During the first year they were maintained in a green house at temperature of 24-30°C, and a 16 hour photoperiod using supplementary lighting when necessary. At the end of that year the plants were moved to another green house at 24-27°C and the same photoperiod, where they were maintained for the rest of the experimental period.

The control of mites, aphids and white flies was carried out by applying Tedion (Midox), Dicofol (Octavius Hunt), Pynosect (Mitchell Cotts Chemicals), Malathion (ICI), Gamma Col (ICI) or X-All-Insecticides (Synchemicals) when required. At times, the red mite infection was so severe that drastic pruning was necessary.

Four pots were allocated to each of the seven genotypes, each of them containing one plant only, this number of plants was sufficient to provide the *in vivo* material for the experiments. New plants were produced every year from vines that originated from the old plants in order to avoid senescence.

2.1.2 Growth and maintenance of in vitro plants

In vitro stock plant cultures were taken from the stock of donor plants maintained in the green house.The herbaceous cuttings of variable size, containing 10-15 axillary buds were collected from these plants and washed in distilled water, then segmented in pieces containing one or two axillary buds which were surface sterilised with a rapid immersion in a 70% ethanol followed by a 15 minutes immersion in 5% sodium hypochlorite and washed 4 times in sterile distilled water. The excision of the meristems was made under a stereo-microscope (Olympus), using a pair of forceps, a hypodermic needle (Sabre International Products Ltd.) and a scalpel.

The genotypes CN-1367-2 and Rose Centennial have larger meristems than Brondal (0.4 cm), Jersey Orange, TIB-10, TIB-9 and Papota (0.3 cm).

All the manipulation of plant material during and after the sterilisation were performed in the sterile environment provided by a laminar flow cabinet (Microflow Pathfinder Ltd.).

Three different nutrient media were used at distinct stages: **a**) MII-m for meristem initiation, **b**) MII-t for the explants which started to develop, and **c**) MII-p to keep the stock plants. The compositions of these media are provided in Table 2.1.

The meristems were cultured in 5 cm disposable petri dishes with 15 ml of MII-m. The stock plant cultures were stored in 100 ml disposable jars containing 30 ml of MII-p. All the cultures were maintained in a culture room at 25±2° C and 16 hour photoperiod with 30 $\mu Mm^{-2}s^{-1}$PAR. *In vitro* stock plants were sub-cultured every 4-6 months, according to the needs of each genotype.

2.2 Nutrient Media for Tissue Culture

The basic media used for the tissue culture experiments were developed for sweet potato at the International Potato Centre (CIP), Peru (Table 2.1) (R. Lizarraga, personal communication). The media were originally based on a full strength of Gamborg's B-5 or half strength of MS (Murashige and Skoog, 1962) salts

with the minimal organic salts (Appendix 1). The tests described in this thesis were based on CIP's media with a half strength of MS.

Unless otherwise stated, the only change was the reduction of sucrose levels from 3 and 5% in the CIP's original media to 2 and 3% in MII-p and MII-m/MII-t, respectively.

Media were heat sterilized in autoclave for 15 minutes at 15 $lb.in^{-2}$(1.87 bar) at 120°C. The media were sterilized in glass jars of variable volumes and poured into the culture vessels at 45°C and allowed to solidify at room temperature. Generally the media were used within a few days.

Table 2.1 Nutrient media for sweet potato (R. Lizarraga, 1986, personal communication).

Compound	MII-m ($mg.l^{-1}$)	MII-t ($mg.l^{-1}$)	MII-p ($mg.l^{-1}$)
MS salts + minimal organics	1/2 strength	1/2 strength	1/2 strength
Calcium Panthotenate	2	2	2
Gibberelic acid	20	15	10
Ascorbic acid	100	100	100
Calcium nitrate	100	100	100
L-arginine HCl	100	100	100
Putrescine HCl	20	20	20
Kinetin	0.4		
Sucrose	30000	20000	20000
Agar	7000	7000	7000
pH	5.6	5.6	5.6

2.3 Environmental Conditions

The majority of the experiments were carried out in a culture room at 25±2^{0}C with 16 hour photoperiod supplied by fluorescent tubes generating 30 $\mu Mm^{-2}s^{-1}$PAR. In some of the experiments these environmental conditions differed from the above, these variations are explained in the relevant sections as they occur.

2.4 General Experimental Procedures

2.4.1 Tissue culture methodology

All tissue culture manipulations were done with a x10 Olympus stereo microscope, using a pair of forceps and a scalpel for general subculturing, a hypodermic needle for excising meristems and a pair of micro pincer scissors for root-tip work. The manipulations of *in vitro* roots were done on a filter paper sheet wet with liquid medium in order to avoid dehydration of the fragile root-tips.

2.4.1.1 Slow growth of nodal segments under oil-overlay

Nodal segments, 0.3-0.5 cm in length, were obtained from *in vitro* stock plants with 8-12 internodes. Three days before the excision the donor plants had their apical meristems removed in order to break the apical dominance.

The segments were sub-cultured in 10 x 1.5 cm disposable test tubes containing 3 ml MII-t without GA_3.

Liquid paraffin (heavy, BDH) and liquid silicone (Down Corning) were heat sterilized for 15 minutes at 15 $lb.in^{-2}$ (1.87 bar) at 120^0C. After cooling to room temperature, 6 ml of the oils were poured into the test tubes in order to cover the surface of the medium and the nodal segments. The test tubes were then stored in the culture room at 25 ± 2^0C or in incubators at specific temperatures, according to the objective of the experiment.

2.4.1.2 Cryopreservation of shoot-meristems and root-tips

Shoot-meristems were obtained from axillary buds of plants growing in the green house and allowed to grow in MII-m at 25^0C and 16 hour photoperiod for three days prior to the freezing.

Root-tips were collected from 10 day-old *in vitro* plantlets which had originated from nodal segments of stock plant cultures. As at this stage the roots are actively growing an initial culture stage was not used, otherwise the tips would have been too big for freezing.

Initially experiments were made to check the toxicity of cryoprotectants on the organs to be frozen. These cryoprotectants were: DMSO, glycerol, mannitol, sucrose, proline, PVP-40 and sorbitol. The cryoprotectants were diluted into MII-m generally at levels varying from 2.5% to 10% (see section 3.2.3.1 for

details). As some of them proved to be toxic, they were excluded from the cryopreservation tests.

The cryoprotectants used for the freezing experiments were sorbitol, PVP-40, DMSO, glycerol and sucrose, alone or mixed for the shoot-tips and DMSO alone for the root-tips (see section 3.2.3.2 for details).

The technique used for cryopreservation was based on that developed by Henshaw and colleagues (1985). The samples were slow-cooled at a rate of 0.5°C.min^{-1} to various temperatures prior to their immersion in liquid nitrogen (LN) at -196°C (see section 3.2.2.2.2 for details).

Thawing was carried out by quickly placing the 2.2 x 0.3 cm carriers with the meristems into 3 ml liquid medium at room temperature, where they were maintained for 30 minutes. The survival rates of shoot-meristems were verified by subculturing in semi-solid MII-m and the root-tips had their viability checked by adding two drops of a 5:1 (w/v) fluorescein diacetate (FDA) solution in acetone (Widholm, 1972) in 5 ml MII-m without growth regulators and observing the activity under an Olympus BHT epifluorescence microscope with 400-490 nm excitation filter, 500-515 nm mirror and 530 nm barrier filter.

2.4.1.3 Root cultures

Two types of experiment involved the use of root culture technique: a) induction of shoot formation and b) electrophoresis of isozymes. Electrophoresis tests used

root culture as a tool to produce root material for analyses. The adventitious root-tips (length 0.3-0.4 cm x diameter 0.1-0.15 cm) which were still covered by the stem epidermis were obtained from plants growing in the green house (section 2.1.1), these explants were excised from the stems under sterile conditions and cultured in liquid MII-m without growth regulators maintained on a shaker at 80 rpm at 25±2°C and 16 hour photoperiod.

The roots used for tests of shoot induction were obtained from the *in vitro* donor plants and then sub-cultured in liquid or semi-solid MII-m without kinetin and GA_3, supplemented with NAA, BA or IAA as it will be described in chapter 4.

2.4.2 Polyacrylamide gel electrophoresis of esterase isozymes

The PAGE (polyacrylamide gel electrophoresis) experiments were separated into two phases: **a**) development of methodology, **b**) characterization of the seven genotypes using the methodology developed.

The first series of tests was carried out using both *in vivo* and *in vitro* plant materials such as shoot-tips, young leaves, nodal segments and roots from *in vivo* cultures and roots and whole plantlets from *in vitro* which were tested individually using three extraction buffers (sections 5.2.2 and 5.3.1), and stained for various enzyme systems (section 5.3.1 and Appendix 5).

In the next series of tests for the characterization of genotypes using *in vitro* roots, the extraction was done using 50 mM Na_2PO_4 added by 6 mM dithyothreitol, pH 7 (Shields *et al.*, 1983). The gels were stained for esterase isozymes (50 ml phosphate buffer 0.1 M pH 7.0, 1 ml 1-naphthyl acetate 5 mM, 1 ml 2-naphthyl acetate 5 mM, 50 mg Fast Blue RR salt (Kahler and Allard, 1970).

The buffer system was non-dissociating discontinuous at pH 6.8, 8.8 and 8.3 for the stacking and resolving gels and reservoir buffers, respectively (Appendix 4).

The electrophoresis procedure was carried out in a cooled vertical slab Protean-II apparatus, connected to a power supply ATTA-AE-3105, adjusted at 30 mA and variable voltage.

After the electrophoresis was carried out various staining methods were tested for different enzyme systems. Then, the gels were washed in distilled water and fixed for 10 minutes in a solution 5:1:4 ethanol:acetic acid:water.

The last step of preparation in the gels was their drying at 60°C in a BIO Rad slab dryer between a sheet of filter paper and another of cellophane.

The enzymatic characterization of genotypes was based on visual analyses of the gels and calculating the relative electrophoretic mobility (Rf= the distance migrated by protein divided by the distance migrated by the dye (Hames and Rickwood, 1981) of the bands.

CHAPTER 3

SWEET POTATO GERMPLASM CONSERVATION

3.1 SLOW GROWTH UNDER OIL-OVERLAY

3.1.1 Introduction

Although the potential of oil-overlay as an alternative for preserving plant cultures has been demonstrated as long as 30 years ago by Caplin (1959), it is only in the last few years that there has been renewed interest in the method for germplasm storage. Reviewing the literature, the technique of oil-overlay has been mentioned with respect to the conservation of callus (Caplin, 1959; Augereau *et al.*, 1986; Moriguchi *et al.*, 1988; Mathur *et al.*, 1991); cell cultures (Mannonen *et al.*, 1990; Mathur *et al.*, 1991) and tissues, including root segments, leaf discs, apical and axillary buds and encapsulated propagules (Mathur *et al.*, 1991). To date, however, there are no reports in the literature on the use of oil-overlay as a method for the storage of vegetatively propagated plant germplasm.

The aim of the experiments presented below was to check **a**) whether it was possible to conserve micropropagated nodal segments of sweet potato under oil; **b**) the effects of the oil on the survival, the growth rates and the viability of explants after storage.

3.1.2 Material and Methods

3.1.2.1 Plant material

Three experiments were carried out to study the technique of oil-overlay conservation. The genotypes used for the first test were Papota, TIB-10 and CN-1367-2.

The experiments were based on twelve replicates of 0.3-0.5 cm nodal segments originated from *in vitro* donor plants which had 8-12 internodes. A pruning of the apical meristem three days before the experiments were initiated was considered enough to break the apical dominance. Each explant consisted of the nodal segment and one axillary bud which were sub-cultured in 10 x 1.5 cm disposable test tubes, where the oil was later added.

The first experiment was carried out by testing three different stages of development (sizes) of plantlets: **a**) ten-day old (0.3-0.9 cm) segments in which the axillary bud was starting to develop; **b**) twenty-day old (1.0-1.9 cm) plantlets in which the axillary bud had developed to form a small shoot with 2-4 young leaves and roots were only starting to develop; **c**) thirty-day old (2.0-3.0 cm) plantlets which were already rooted and with the axillary shoot developed to the 5-7 leaf stage.

The second experiment tested the effects of oil-overlay replicates of two types of explants: ten-day old plantlets with the axillary buds starting to develop (the sizes varied from 0.3 to 1.0 cm) and newly sub-cultured nodal segments with no development (0.3 to 0.6 cm).

The third experiment had LP and LS added immediately after the nodal segments with no development (0.3 to 0.6 cm) were sub-cultured and tested their development at 15°C, 20°C, 25°C and 30°C.

3.1.2.2 Methods

The oils that were used in these experiments were liquid paraffin (LP) and liquid silicone (LS). Both oils were heat sterilized, cooled to room temperature then they were poured over the explants forming a 6 cm column over the semi-solid MII-t nutrient medium.

The first and second experiments were maintained at 25±2°C with 16 hour photoperiod for 17 and 10 months, respectively. The third test was carried out at 15°C, 20°C, 25°C and 30°C with 16 hour photoperiod for 8 months.

Observations were made initially at weekly intervals, when rapid changes were occurring in the explants; later observations were made on a monthly basis. The data collected on these occasions were observations dealing with size of the plantlet, number of

axillary shoots, leaves and adventitious roots, death of shoots and leaves and presence of callus.

The final plant measurements of the first experiment were made after 12 months and again after 17 months when the recovery growth test was also carried out. However, as the growth results at 12 months did not differ significantly from those at 17 months, the full analysis was carried out on the 12 month results only.

The recovery of explants after conservation was tested by subculturing the nodal segments using the normal sweet potato micropropagation procedure (see section 2.1.2), with both liquid and semi-solid (0.7% agar) MII-t nutrient media. The semi-solid medium was contained in 9 cm disposable petri dishes wile the liquid medium was in 50 ml disposable jars. After two weeks all of the explants were sub-cultured onto semi-solid medium and the recovery growth was determined by the ability of the explants to grow over a period of one month.

3.1.3 Results

3.1.3.1 Effects of LP and LS overlay on the development and storage of sweet potato cultures

The use of both LP and LS overlay produced plantlets with very short internodes and many axillary shoots (Plates 3.1.1-a,c; 3.1.3-a), and increased the interval between sub-cultures to 12-17 months (Figures 3.1.1, 3.1.2, 3.1.3).

The use of both oils increased the period of survival of explants, but LP proved to be more efficient for the long-term storage of the three genotypes selected. Some of the differences in response affected the survival of explants (see Figure 3.1.3) and others affected the pattern of development (see Figures 3.1.1 and 3.1.2), as outlined below.

One week after the oil addition, the more intensely green coloured genotypes (Papota and TIB-10) showed a strong fading in their pigmentation. The oil-overlay also killed the shoot-tips of some of the type **b** and type **c** explants (see section 3.1.2.1). This reaction was first seen with LS treatments, however it was soon observed in the LP-treated explants. The speed of this response was genotype-specific, however all the genotypes were affected after four weeks. Generally the affected explants developed lateral shoots after this reaction.

Some of the type **c** explants grew to the extent that they emerged above the oil surface; in a few of these explants the area exposed above the oil surface died soon after emergence. Sometimes the whole branch eventually died, thus causing the death of the explant if a new shoot had not developed from the base. With all of the tested genotypes some of the type **c** explants grew out of the oil surface, independent of the oil used. When leaves were produced out of the oil they were normal

in shape, darker, not translucent, and larger than the ones produced under the oil.

All of the leaves produced under the oil were slightly translucent. The leaves produced before the oils were added also became translucent but they soon died, although the axillary buds and stem remained alive. The leaves produced under oil were of reduced size, many times to the extent of loosing their typical leaf shape, turning to a small scale-like organ (Plate 3.1.1-a). The reducing effect on the leaf size which strongly affected Papota and TIB-10 was not so dramatic in CN-1367-2 where leaves were reduced in size but maintained their leaf shape. Some of the smaller leaves produced by CN-1367-2 were oblong with a narrow but long lamina, the petioles were too long for their lamina (Plate 3.1.3-a).

The explants growing under oil developed more axillary buds than the controls. The axillary buds started to develop within a month of the oils being added and explants treated with LP were the first to develop the axillary buds. These shoots were very small with short internodes and minute leaves. The axillary shoots were developed not only from the main stem but also from the newly developed axillary shoots which, despite being very short, already possessed a number of internodes (Plate 3.1.1-c). The proliferation of axillary shoots under the oil-overlay was genotype specific and explants treated with LP were more affected than the ones treated with LS. Figure 3.1.1 shows the production of axillary shoots after 12 months storage under oil-overlay, some of

those shoots produced during the treatment were already dead, but most of them were still alive.

A few of those axillary shoots were malformed: some had leaves which did not develop, looking like scars along the very short stem, whereas others were conical shaped with a large base and the meristematic region standing out of the stem forming structures which were elongated or rounded like a small mushroom dome, and a few were albino (Plate 3.1.2-a,b,c).

The majority of the larger (type **c**) and medium (type **b**) explants (see section 3.1.2.1) produced had their first roots before the oil treatments, and during the conservation period they had better root systems than the small and medium sized explants which developed their roots later. Most of the explants which only produced roots after oil treatments, developed a few short lateral roots with bent tips. A few of the explants in all treatments under oil produced a few "nodes" along the roots; when these "nodes" grew on the distal portion of the main root, various short roots could grow from them .

Some of the CN-1367-2 explants produced thick (0.2 cm against the normal 0.05 cm) and very long greenish roots. This reaction occurred in medium size explants (58% of the explants under LP and 16% under LS) and in big size explants (25% of the explants under LP). Generally explants which developed those thick roots did not produce more than one root. No thick roots were produced from small explants which showed very poor rooting: 42% of them did not produce roots. No thick

roots were produced by the controls. Both thick roots and oblong leaves were only observed on CN-1367-2 explants.

Most of the explants developed adventitious roots; this effect started by the second month and increased with time. The adventitious roots grew from the main stem (type **a**) see the arrow **a** on Plate 3.1.1-d) and also from the newly developed axillary shoots (type **b**) (see the arrow **b** on Plate 3.1.1-d). Most of these adventitious roots grew towards the oil surface but did not grow more than few centimetres in length, although some of those produced from the main stem grew into the medium producing lateral roots and apparently behaving as normal absorbing roots. The number of adventitious roots was genotype specific and generally, plantlets covered by LP produced more adventitious roots than plantlets covered by LS and the controls.

The adventitious roots from either the stem or the newly developed shoots could develop either positive or negative geotropism. Parts of some of the normal roots also grew towards the oil surface after growing into the medium for some period. Explants treated with LP tended to produce more roots with a negative geotropism than those treated with LS. A few of the adventitious roots grew out of the oil surface and died soon after emerging.

Some of the explants growing under oil developed a thin layer of callus along their stems. These calli started from isolated points and at the beginning looked like multiple translucent hairs (Plate 3.1.1-b). Both oil

treatments induced calli, but LS seems to induce more than LP, however there was a genotypic specificity; CN-1367-2 explants growing under LS developed very few calli along the stem epidermis, but there was no callusing under LP. Papota and TIB-10 developed more callus under LS than LP, but the callusing was more intense on TIB-10. A few of the controls of all genotypes also developed a few calli along the epidermis.

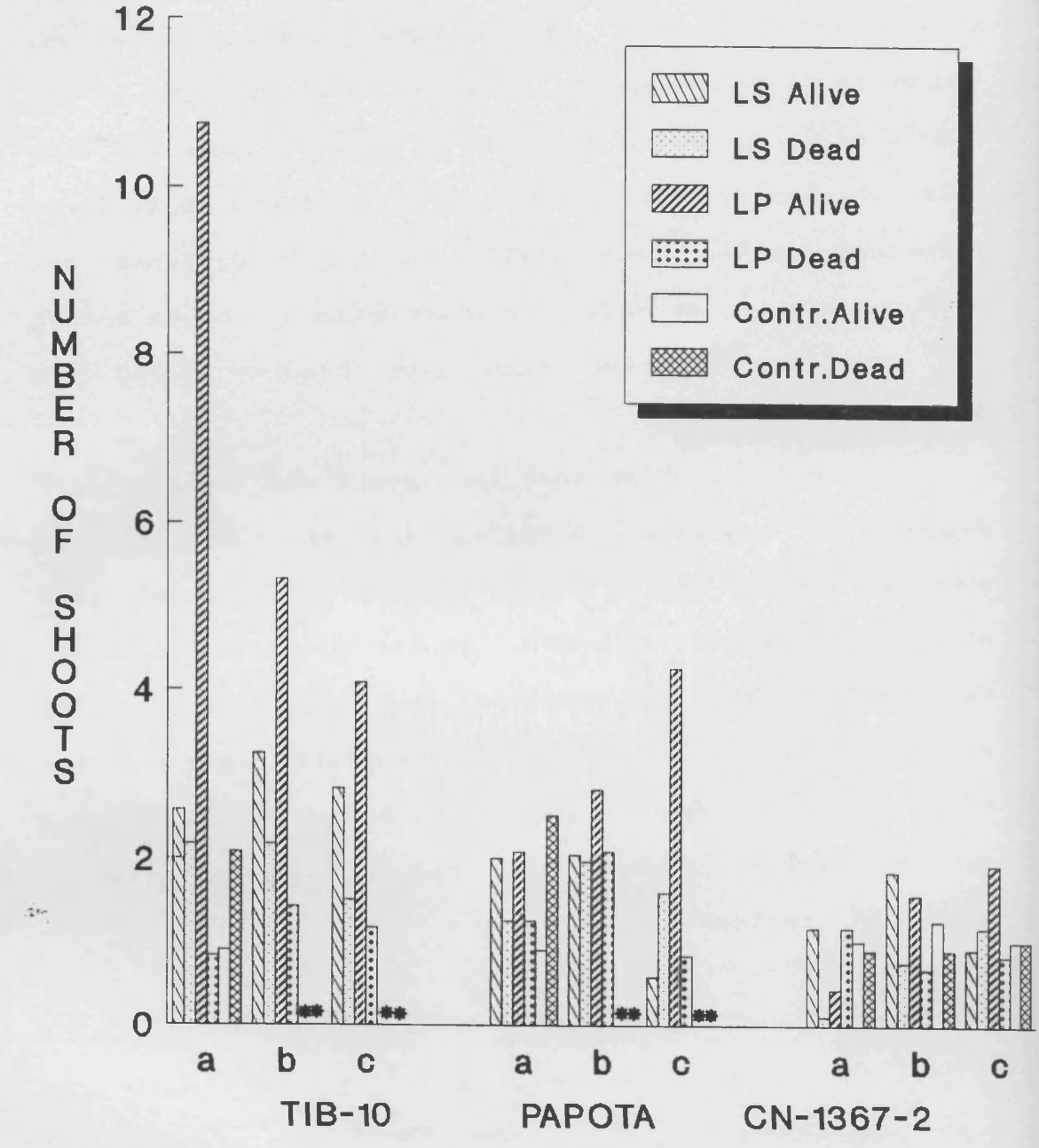
Most of the explants from the controls grew at a normal rate (see Figure 3.1.2), reaching their maximum stage of development and starting their senescence more rapidly than the explants growing under oil, usually as a result of dehydration. The controls of TIB-10 and Papota explants types **b** and **c** (medium and large, respectively) also presented a normal growth rate , but they were already dead at 12 months when final growth data were collected.

CN-1367-2 seemed to have the growth stimulated by LP, as the average lengths for any of the explant categories were higher under this treatment than under others (Figure 3.1.2).

There was some variation in the development of each of the explant sizes according to the genotype, but generally, the larger the explant, the greater was the number of normal leaves formed. The smaller the explant, the greater were the numbers of reduced leaves and nodes per length. TIB-10 was the genotype which best produced non-adventitious shoots and its medium (type **b**) and small (type **a**) explants developed greater numbers of non-

adventitious shoots, which provided a better basis for the survival of this genotype. Most of the small (type a) CN-1367-2 explants did not develop under oil treatments or control treatments, but they were alive at 12 months, and even at 17 months some were still alive, though small (see Figures 3.1.2 and 3.1.3). Most of the TIB-10 explants which survived 17 months under oil were also originally from the small category but these developed better than small CN-1367-2 explants and produced one to several non-adventitious shoots during the process of conservation (see Figures 3.1.3 and 3.1.1).

Figure 3.1.3 shows that the small and medium sized explants of genotype CN-1367-2 and small explants of genotype TIB-10 had a better survival than other types of explants after 17 months growth under oil-overlay. Papota which was the genotype that produced the most explants growing out of the oil surface, was also the first in which death occurred; despite having a good survival rate at 12 months it had no survival after 17 months of treatment.



Legend: a,b and c are types of explants
* plantlets were dead before 12 months

Figure 3.1.1 Shoot production in sweet potato genotypes during storage for 12 months under LP and LS overlay.

Key:

Number of replicates: 12

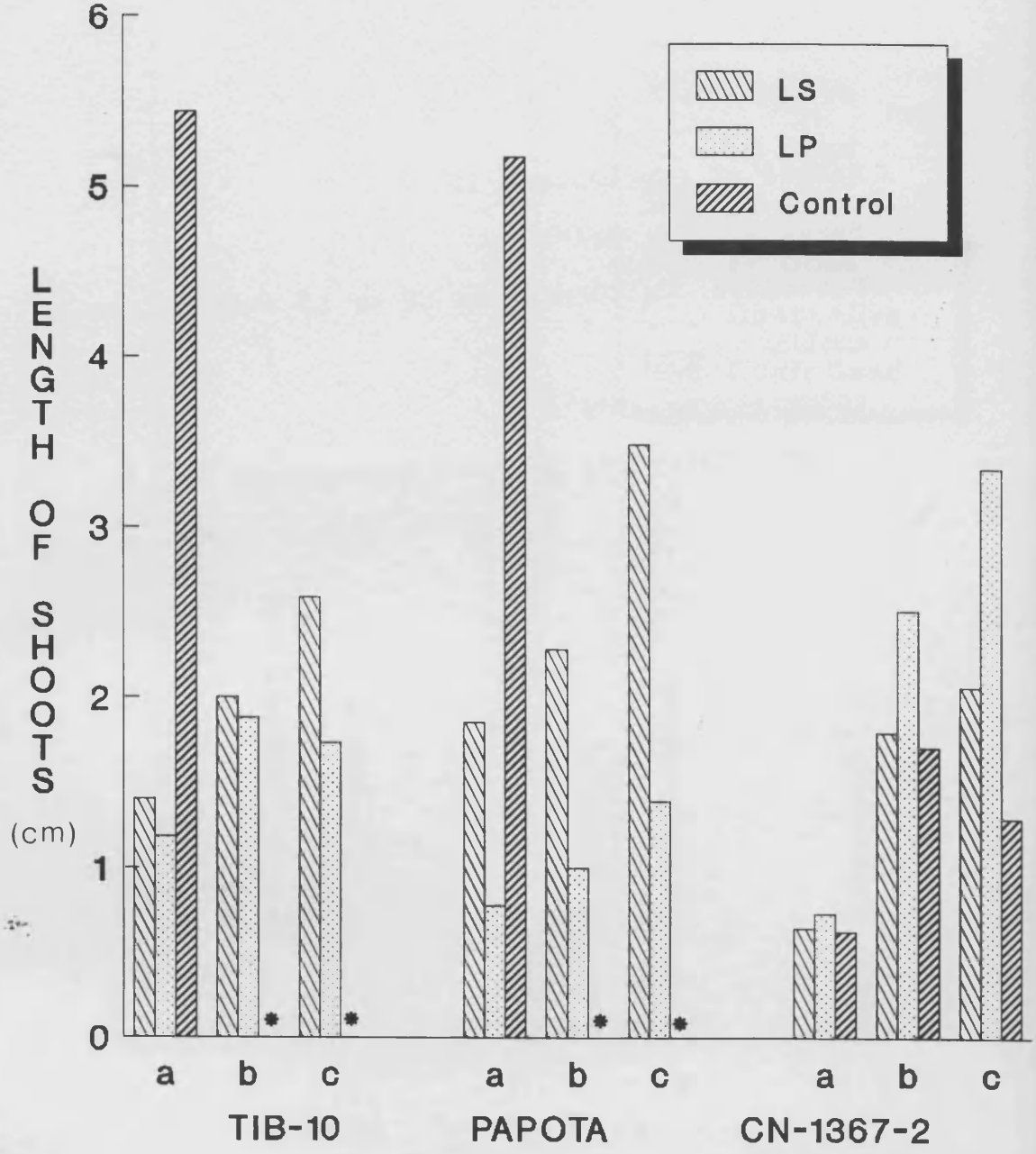
Basal medium: MII-t

Procedure: Culture under LP or LS overlay for 12 months

Temperature: $25\pm2^{0}C$

Light conditions: 16 hour photoperiod

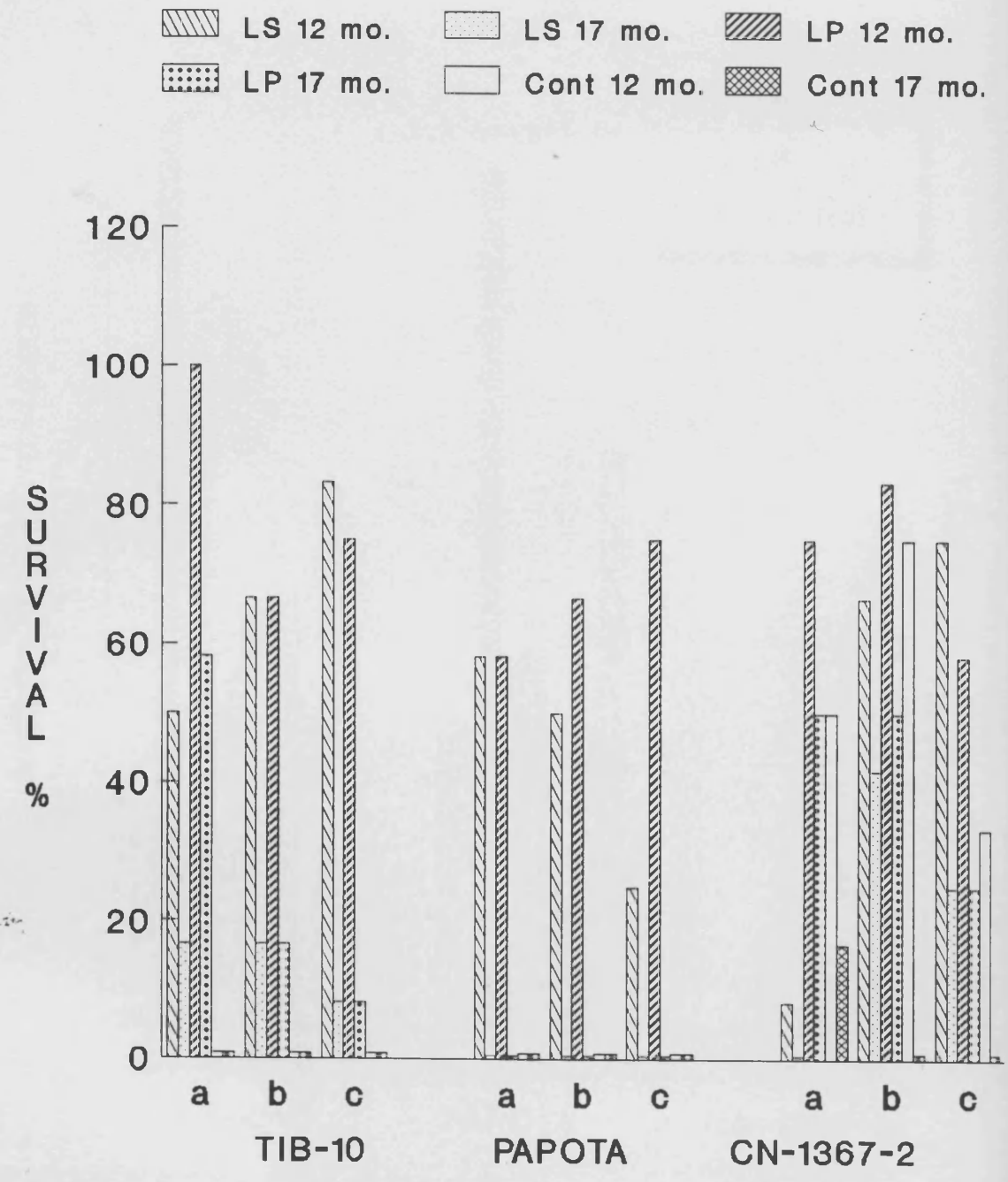
30 $\mu Mm^{-2}s^{-1}PAR$



Legend: a,b and c are types of explants
* plantlets were dead before 12 months

Figure 3.1.2 Growth of sweet potato genotypes during storage for 12 months under LP and LS overlay.

Key: See details in Figure 3.1.1



Legend: a,b,and c are types of explants

Figure 3.1.3 Survival of sweet potato genotypes stored for 12 and 17 months under LP and LS overlay. Note: data were collected at 12 and 17 months

Key:

Number of replicates: 12

Basal medium: MII-t

Procedure: Culture under LP or LS overlay for 17 months

Temperature: $25\pm2^{0}C$

Light conditions: 16 hour photoperiod

30 $\mu Mm^{-2}s^{-1}PAR$

Plate 3.1.1

a

b

c

d

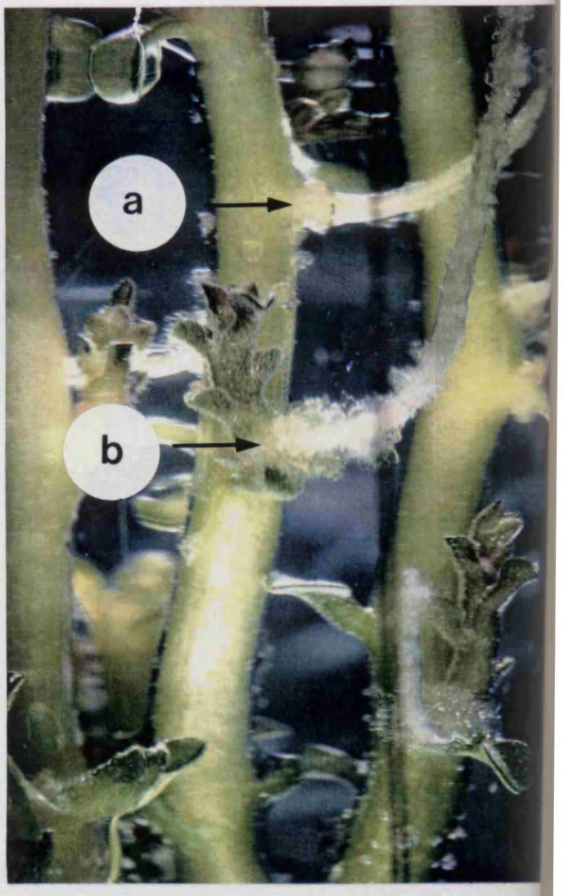


Plate 3.1.1 Details of plantlets maintained under oil overlay at 25± 2^{0}C. Note the genotype specificity in the reaction to the treatments, most obvious in the miniaturing of plantlets; genotype TIB-10 and genotype Papota plantlets show shorter internodes and much more reduced leaves than genotype CN-1367-2.

a) genotype TIB-10 plantlet maintained under LS showing very short axillary shoots with scale-like leaves.

b) genotype Papota plantlet maintained under LS with callus starting to grow from isolated points like multiple translucent hairs.

c) genotype TIB-10 plantlet maintained under LP showing various diminished shoots, some of them originating from newly developed sprouts.

d) Stems of genotype CN-1367-2 showing adventitious roots growing from the stem (a) and from the axillary shoot (b). Note also the negative tropism of both types of adventitious roots.

Plate 3.1.2

a

b

c

Plate 3.1.2 Abnormal axillary shoots produced from plantlets stored under oil overlay at 25±2^{0}C.

a) genotype TIB-10 plantlet stored under LP showing conical shoots with leaves reduced to scars and elongated meristems.

b) Detail of a genotype TIB-10 plantlet stored under LS showing albino shoots.

c) Detail of a genotype TIB-10 plantlet stored under LP showing a conical shaped shoot with a prominent meristem looking like a small mushroom dome.

3.1.3.1.1 Recovery of explants after 17 months storage under LP or LS

The final phase of this experiment was to check the viability of the cultures stored for 17 months under oil-overlay. It was a simple but clear proof of the efficiency of the method.

The axillary shoots and nodal segments from the live cultures (see Figure 3.1.3) were sub-cultured to MII-t medium, both in liquid and semi-solid form. Papota cultures did not survive until 17 months, thus only CN-1367-2 and TIB-10 could be tested. The amount of shoots and nodal segments available for the test varied according to the responses of the genotype to the different treatment and in some cases the number of explants tested was very reduced (see Figures 3.1.4, 3.1.5-A and 3.1.5-B).

The recovery of explants after 17 months under oil-overlay was quite successful and some of the explants quickly developed both roots and leaves which were very similar to the original donor plants (Plate 3.1.5-a). However, there was a great variation on the development of explants maintained under the same treatments (Plate 3.1.3-b); many of the explants were still in a lag phase one month after the sub-culture; these explants remained green but did not grow within a month (Figures 3.1.4, 3.1.5-A and 3.1.5-B).

Liquid medium seems to be more satisfactory than semi-solid medium for the initial phase of recovery, as

it induced the explants to grow more rapidly, although it also induced great elongation in the explants from both TIB-10 and CN-1367-2 (Plate 3.1.4-a,b,d). There was a large variation in the extent of this effect, from plantlets that grew a few centimetres more than the normal to others which grew up to 10 cm in length with only 3-4 nodes. This effect was more common in explants originating from LS treated plants. In some of the explants the elongation occurred also in the leaves so that they had both very long petioles and laminas (Plate 3.1.4-d). One of the elongated plantlets from TIB-10 grew to about 5 cm long with no visible leaves, nodes or roots; another from CN-1367-2 was even longer and only developed a single long adventitious root (Plate 3.1.4-a). The elongated plantlets, however, developed axillary shoots with normal internodes when transferred to semi-solid medium (Plate 3.1.4-b). Generally explants growing on semi-solid medium developed normal shaped leaves which were chlorotic at first but soon recovered the pigmentation (Plate 3.1.3-b).

With CN-1367-2, two of the controls were alive after 17 months, but they were both in quite a severe state of dehydration. The sub-culture of these plantlets was carried out by using 2-3 node cuttings which had quite short internodes, and most of these explants produced multiple shoots without a defined dominance between the original nodes, however, not all of them had developed roots within a month.

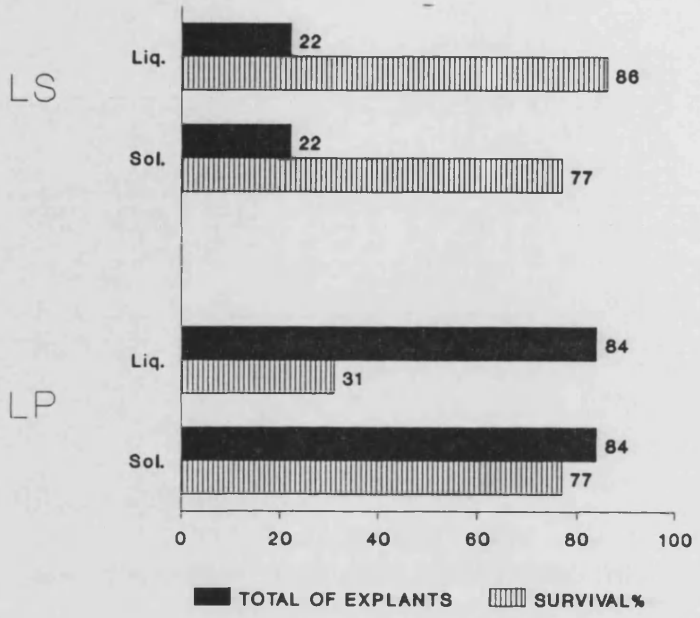
The apical dominance which was broken during the conservation under oil seemed to persist after the subculturing, as the production of multiple shooting was observed in some explants growing on either semi-solid or liquid medium (Plate 3.1.4-c,d).

The first leaves produced after the sub-culture were still translucent (Plate 3.1.4-d), but this effect eventually disappeared and the next leaves to grow were normal (Plate 3.1.4-b), recovering the characteristic shape and colour of the genotypes.

There was a bacteria contamination which killed some of the explants. The bacteria had possibly been latent within the tissues since they developed also in some of the media during the oil-overlay conservation, killing the roots and eventually the explants.

A few of the explants developed some callus on their base (Plate 3.1.4-c), however none of the plantlets were originated from those calli.

SURVIVAL



PHASES OF DEVELOPMENT

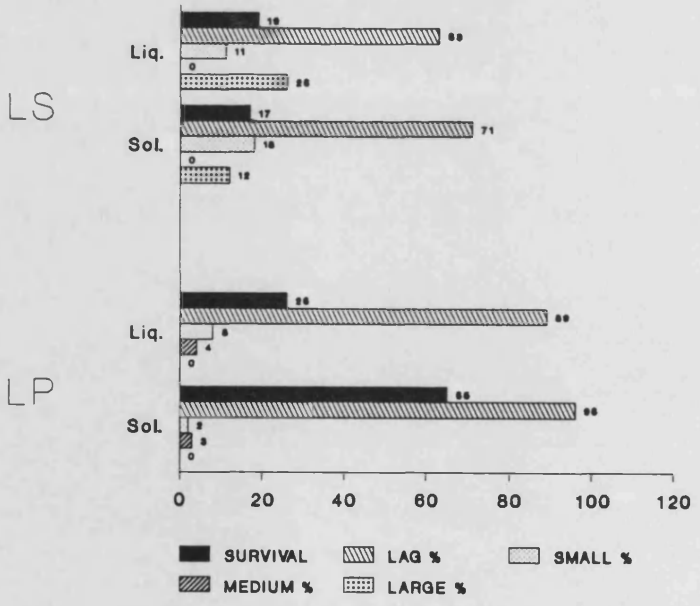


Figure 3.1.4 Survival and development of TIB-10 explants subcultured after 17 months oil-overlay storage

Key:

Number of explants: variable

Basal medium: MII-t (liquid and semi-solid)

Procedure: micropropagation of shoots and nodes on MII-t (liquid or semi-solid) for 2 weeks and then sub-culture to semi-solid MII-t

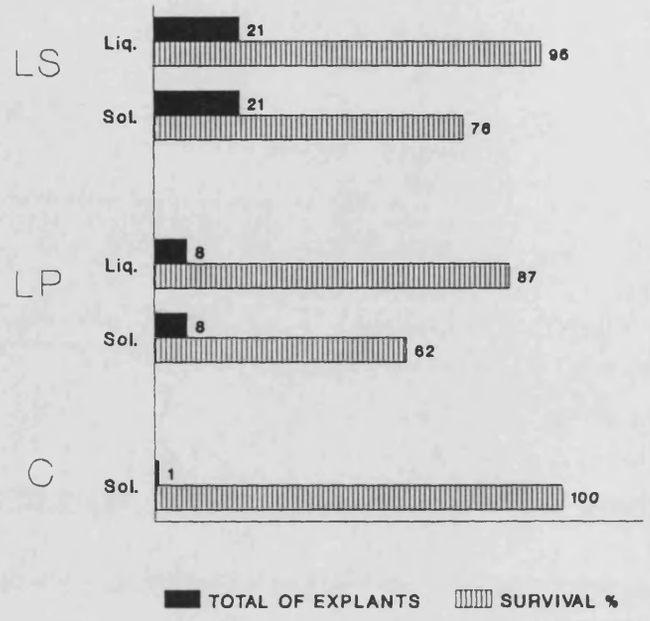
Period of culture: 1 month

Temperature: $25\pm2^{0}C$

Light conditions: 16 hour photoperiod

30 $\mu Mm^{-2}s^{-1}PAR$

SURVIVAL



PHASES OF DEVELOPMENT

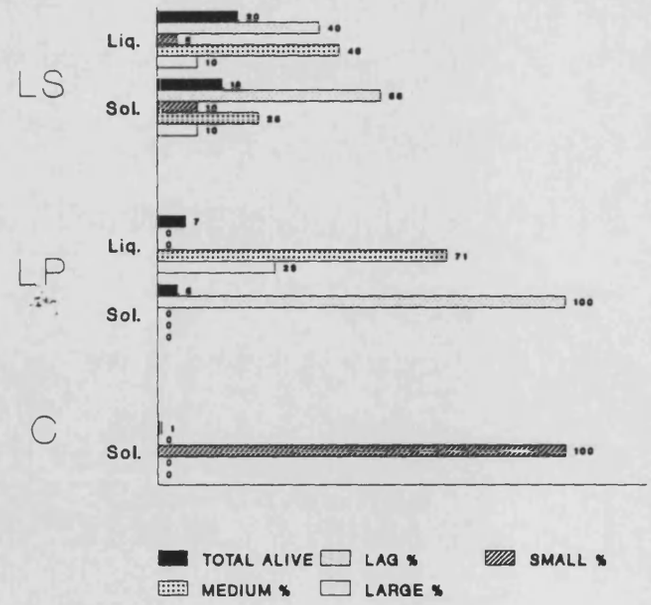
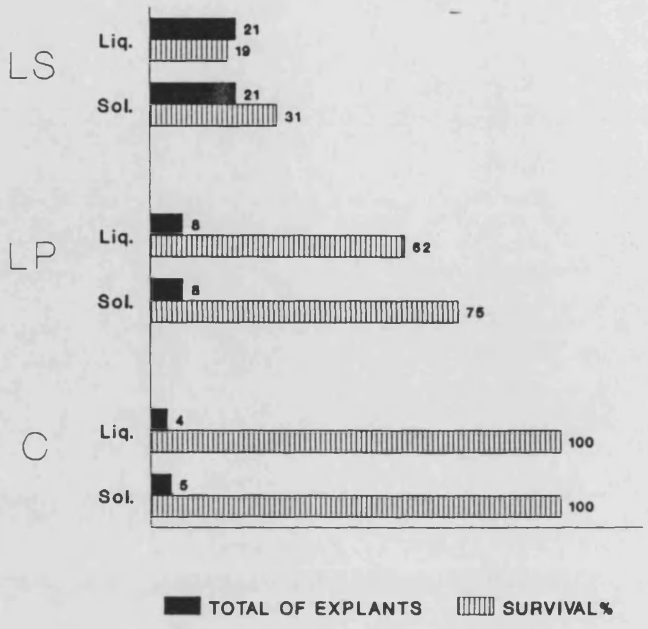


Figure 3.1.5-A Survival and development of CN-1367-2 shoots subcultured after 17 months oil-overlay storage

Key: See details in Figure 3.1.4

SURVIVAL



PHASES OF DEVELOPMENT

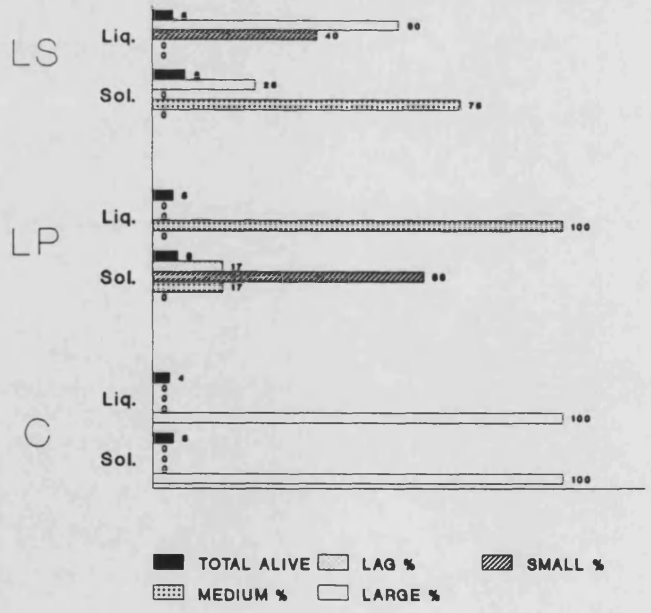
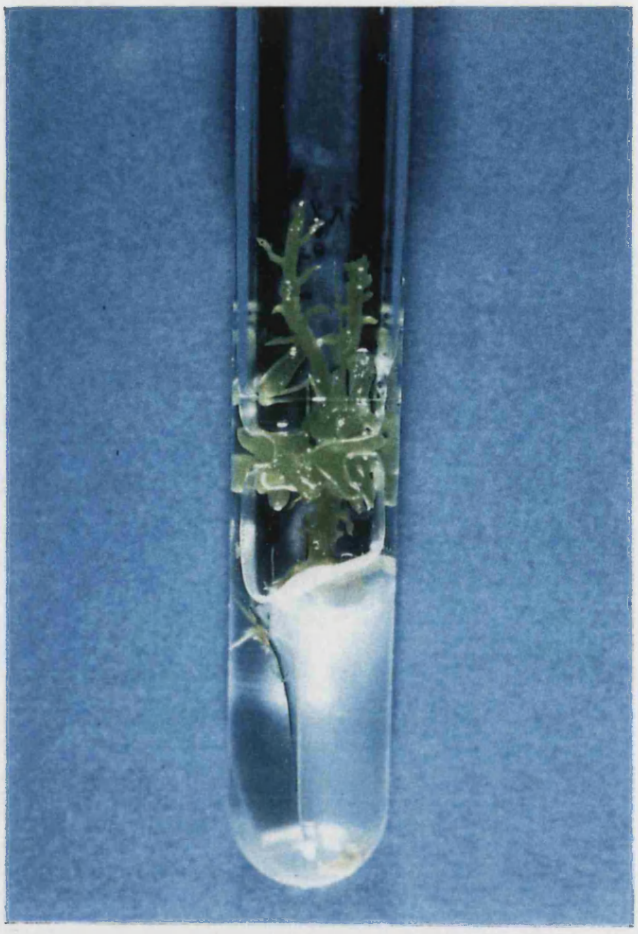


Figure 3.1.5-B Survival and development of CN-1367-2 nodal segments subcultured after 17 months oil-overlay storage

Key: See details in Figure 3.1.4

Plate 3.1.3

a



b

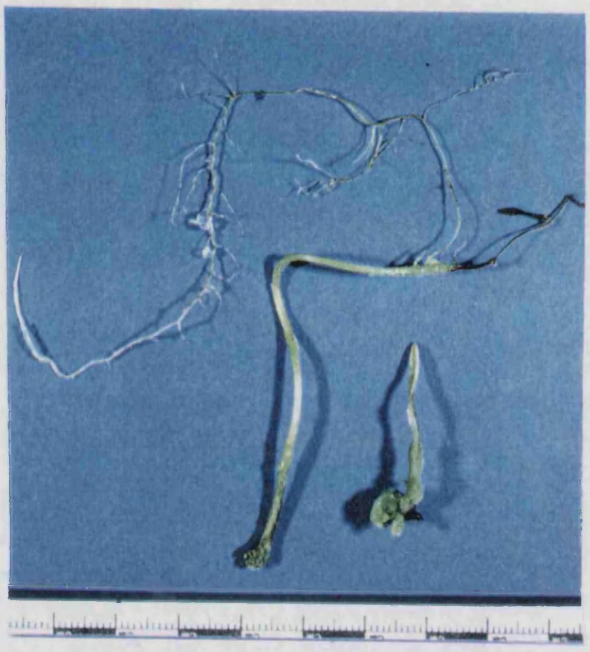
Plate 3.1.3 Minimal growth conservation: the storage of genotypes using oil overlay for 17 months.

a) genotype CN-1367-2 plantlet stored under LP. The larger leaves at the base were produced before the oil addition.

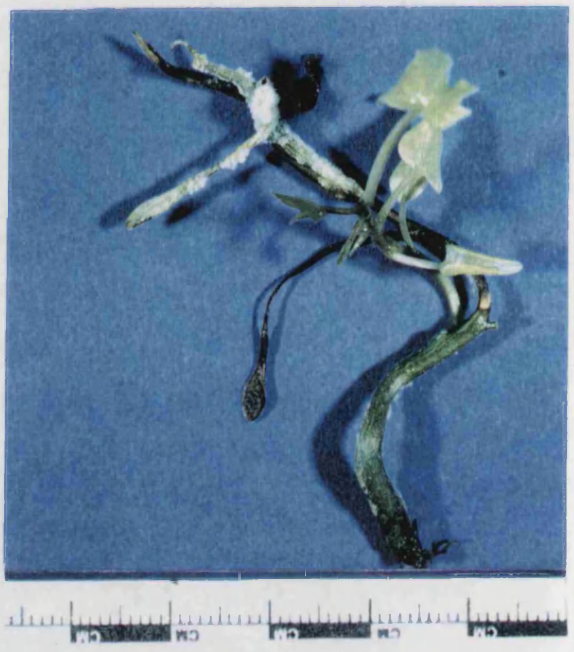
b) Recovery of axillary shoots of genotype TIB-10 on semi-solid MII-t after being stored under LP for 17 months. Note: a) the nonuniformity on the development of the shoots, b) the chlorosis and translucence of the older leaves.

Plate 3.1.4

a



b



c

d

Plate 3.1.4 Regeneration of axillary shoots after being stored under oil overlay for 17 months.

a) Malformed elongated shoots recovered from LS on liquid medium; the left one (genotype CN-1367-2) only developed a long adventitious root with secondary growth; the other one (TIB-10) did not develop leaves, nodes or roots.

b) Very elongated shoot of genotype CN-1367-2 showing a normal sprout after been transferred from liquid to semi-solid MII-t. Note the elongated dead leaf on the base of the new shoot which was produced in liquid medium.

c) Minute multiple sprouts growing from the apex of a genotype TIB-10 shoot recovered from LP on semi-solid MII-t.

d) Well-developed multiple sprouts growing from a genotype CN-1367-2 shoot regenerated on liquid MII-t.

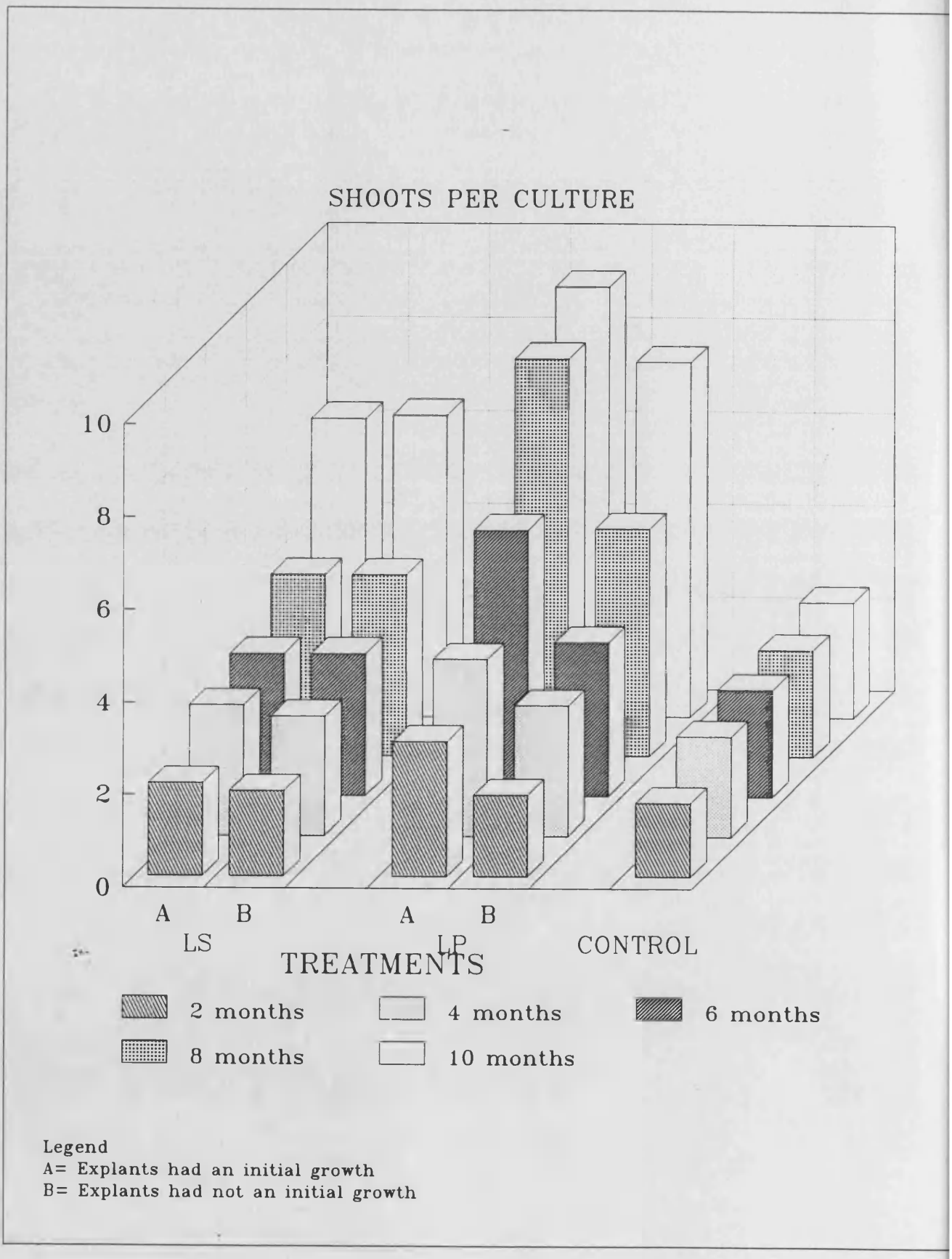
3.1.3.2 Effects of initial growth phase on the conservation under LP and LS

The second experiment was based on the results obtained from the previous tests.

As CN-1367-2 although had previously slower responses to the technique which were similar to those of Papota and TIB-10; it was not considered appropriate to be used for the remaining experiments because of the longer periods that would have been required to obtain results. This meant, therefore, that this genotype was particularly suited to those storage conditions.

The previous tests also showed that the response of explants at the initial stages of development (small or medium) was better than of that explants at a more advanced stage, it was, therefore, important to define which of the initial stages could give the best response for the oil-overlay storage technique and also to determine if the initial phase of growth was critical. The explants were divided into two groups: the first group was allowed 10 days of growth from the time of sub-culture to the moment of adding the oil-overlay; the second group had the oil-overlay added immediately after the sub-culture (see sections 3.1.2.1 and 3.1.2.2).

The general effects of LP and LS on the explants followed the same pattern as in the previous oil-overlay experiment; also the effects on the genotypes followed a similar pattern.



Legend
A= Explants had an initial growth
B= Explants had not an initial growth

Figure 3.1.6 Effects of initial growth phase on the production of axillary shoots of TIB-10 genotype stored for 10 months under LP and LS overlay

Key:

Number of explants: 12

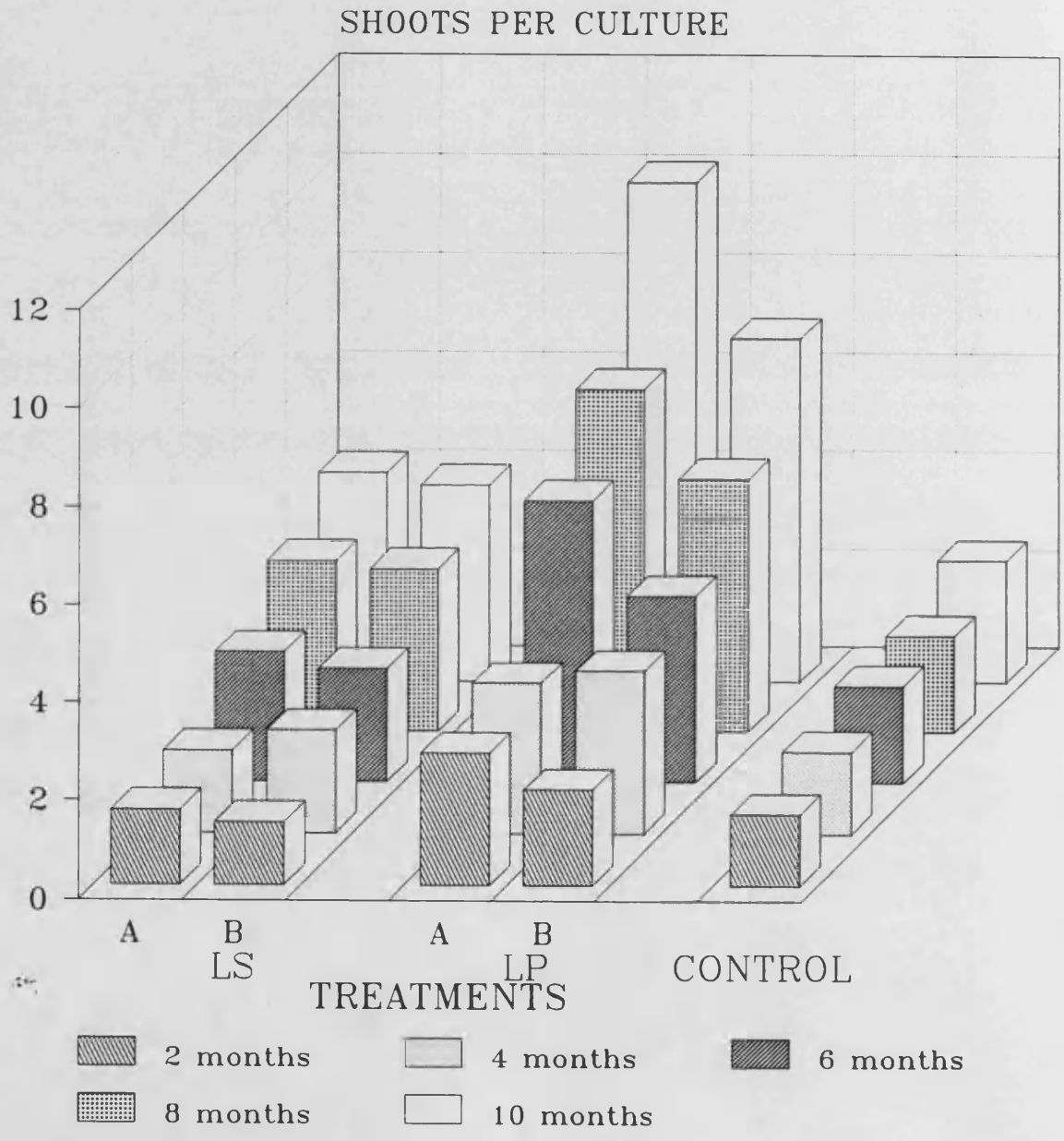
Types of explants: A) 10-day old plantlets
B) nodal segments immediately after the sub-culture

Basal medium: MII-t

Procedure: Culture of the explants under LP and LS overlay for 10 months

Temperature: $25\pm2^{0}C$

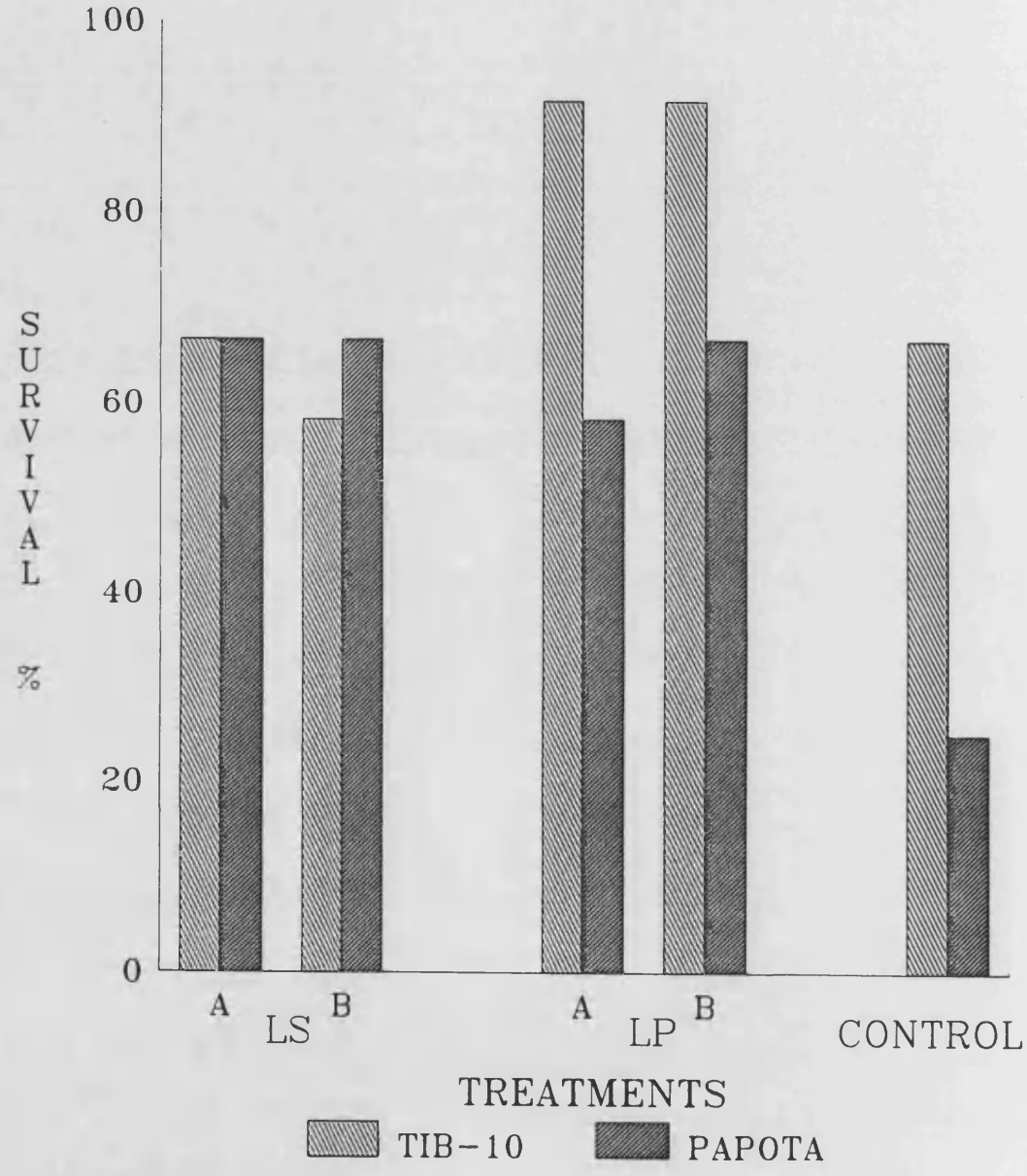
Light conditions: 16 hour photoperiod
30 $\mu Mm^{-2}s^{-1}PAR$



Legend
A= Explants had an initial growth
B= Explants had not an initial growth

Figure 3.1.7 Effects of initial growth phase on the production of axillary shoots of Papota genotype stored for 10 months under LP and LS overlay

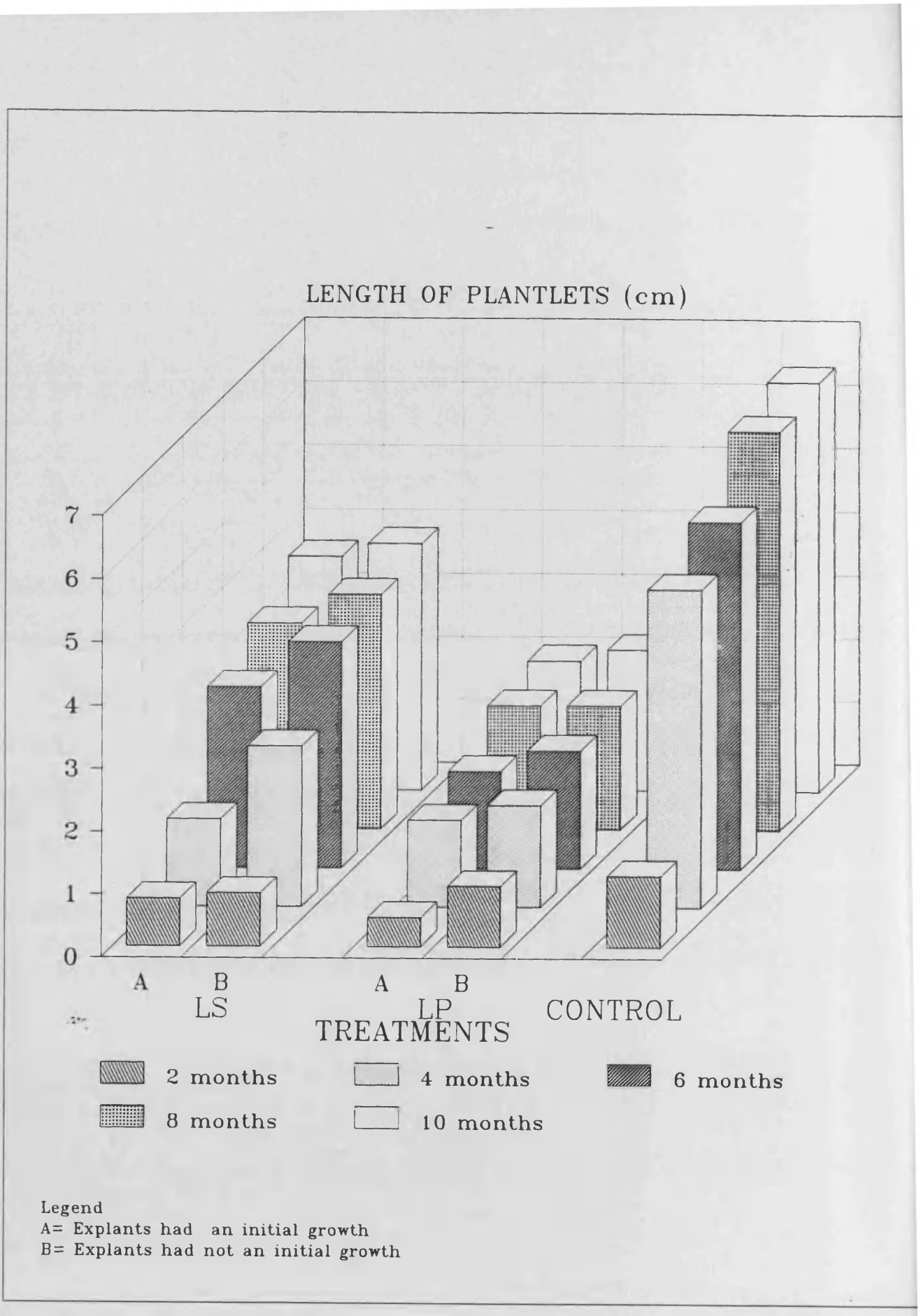
Key: See details in Figure 3.1.6



Legend
A= Explants had an initial growth
B= Explants had not an intial growth

Figure 3.1.8 Effects of initial growth phase on the survival of TIB-10 and Papota genotypes stored for 10 months under LP or LS_overlay.

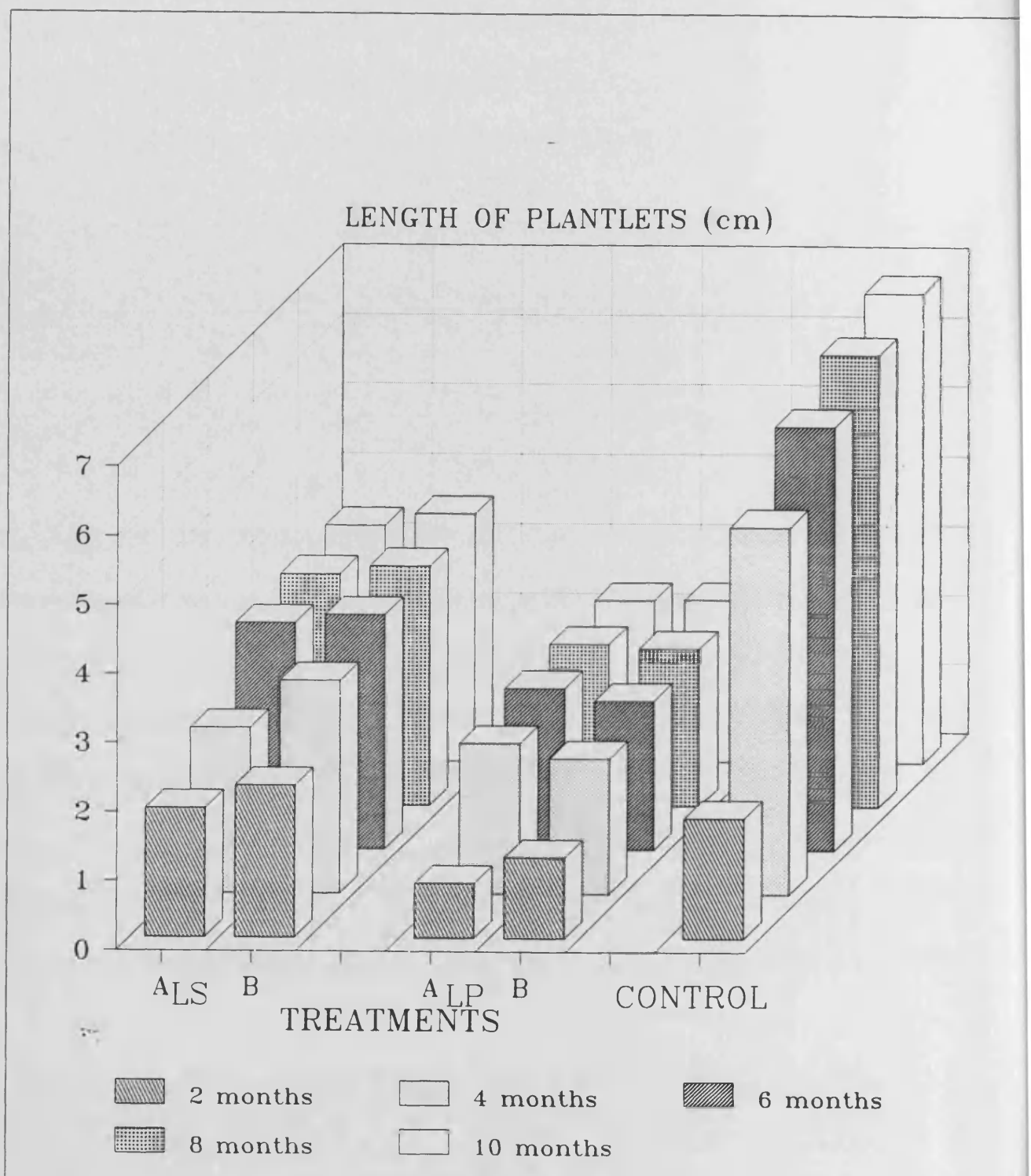
Key: See details in Figure 3.1.6



Legend
A= Explants had an initial growth
B= Explants had not an initial growth

Figure 3.1.9 Effects of initial growth phase on the growth of TIB-10 genotype stored for 10 months under LP and LS overlay.

Key: See details in Figure 3.1.6



Legend
A= Explants had an initial growth
B= Explants had not an initial growth

Figure 3.1.10 Effects of initial growth phase on the growth of Papota genotype stored for 10 months under LP and LS overlay.

Key: See details in Figure 3.1.6

3.1.3.3 Effects of four temperatures on the storage of sweet potato cultures under LP and LS overlay

The third experiment was based on the results from the first and second assays, it was designed to test the effects of four temperatures (15°C, 20°C, 25°C and 30°C) on the conservation of newly sub-cultured nodal segments.

The results were collected after 8 months storage because time restrictions did not permit the further continuation of the experiment; at that time there were no major differences between the effects of the treatments on survival, and the plantlets growing in the control conditions and under the oil-overlays were all in good conditions. The effects of temperature on the development of explants were, however, quite clear (Figures 3.1.11, 3.1.12, 3.1.13), eventhough, the final effects on survival could not be registered. Explants growing under the oils had their development retarded, following the same pattern as in the two previous experiments; both oils reduced the development of the explants, but the effects of LP were greater than LS.

The lowest temperatures of 15°C proved to be unsatisfactory for the conservation of the cultures; a few explants grew only a few centimetres, producing no more than one shoot, and they appeared chlorotic, weak and loosing the turgor. Most of the death at this temperature occurred around the 7-8th months and cultures under LP were in worse condition than either cultures under LS or the controls.

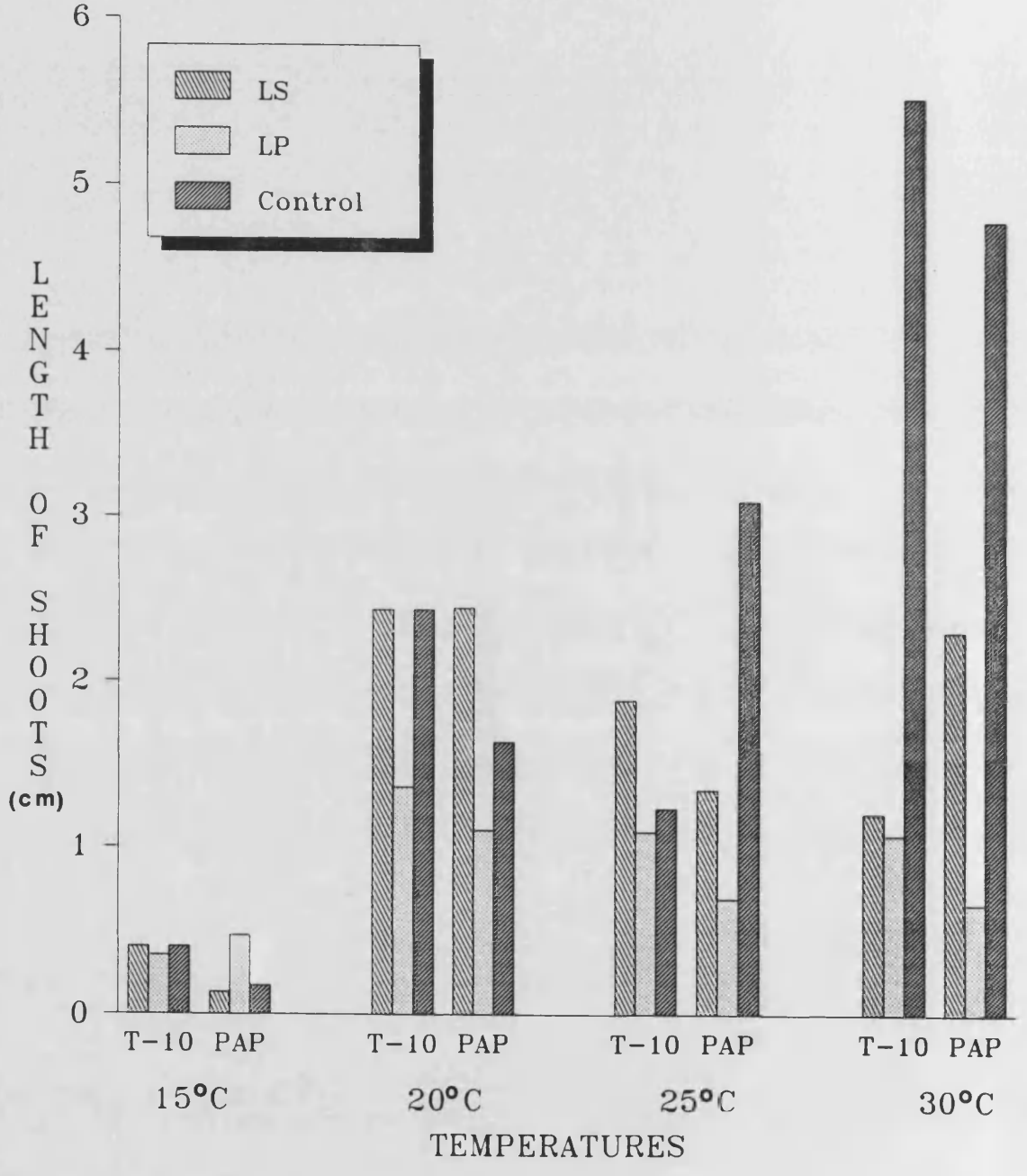
Both the cultures covered by the oil-overlay and the controls showed the best results when stored at 20°C, where the plantlets were very healthy. However, the cultures of both Papota and TIB-10 growing under control conditions had better survival rates than the cultures growing under the oil-overlay (Figure 3.1.13).

The survival rates of cultures of both genotypes under LP were similar at 20°C and 25°C and better than at any other tested temperature, and the development of axillary shoots was also greater. The plantlets at 25°C, however, did not look as healthy as those maintained at 20°C.

Although TIB-10 explants growing under either LP or LS overlay developed a greater number of axillary shoots at 30°C than at any other tested temperature, only LP had the same effect on Papota at this temperature. The best axillary shoot production from Papota explants was at 25°C under either LP or LS, although LP generally, induced the best axillary shoot production at any temperature other than 15°C, where both Papota and TIB-10 produced no axillary shoots (Figure 3.1.12).

The growth rates of the controls increased with the increase in temperature up to 30°C. The development of axillary shoots on cultures under the oil-overlay was stimulated by this temperature, but the plantlets were not very green and healthy and Papota cultures did not survive for a long period.

The latent bacteria which developed at a late stage in the two previous experiments were observed once again.



Legend: T-10= TIB-10 / PAP= Papota

Figure 3.1.11 Effects of four different temperatures on the growth of sweet potato genotypes stored for 8 months under LP and LS overlay

Key:

Number of replicates: 12

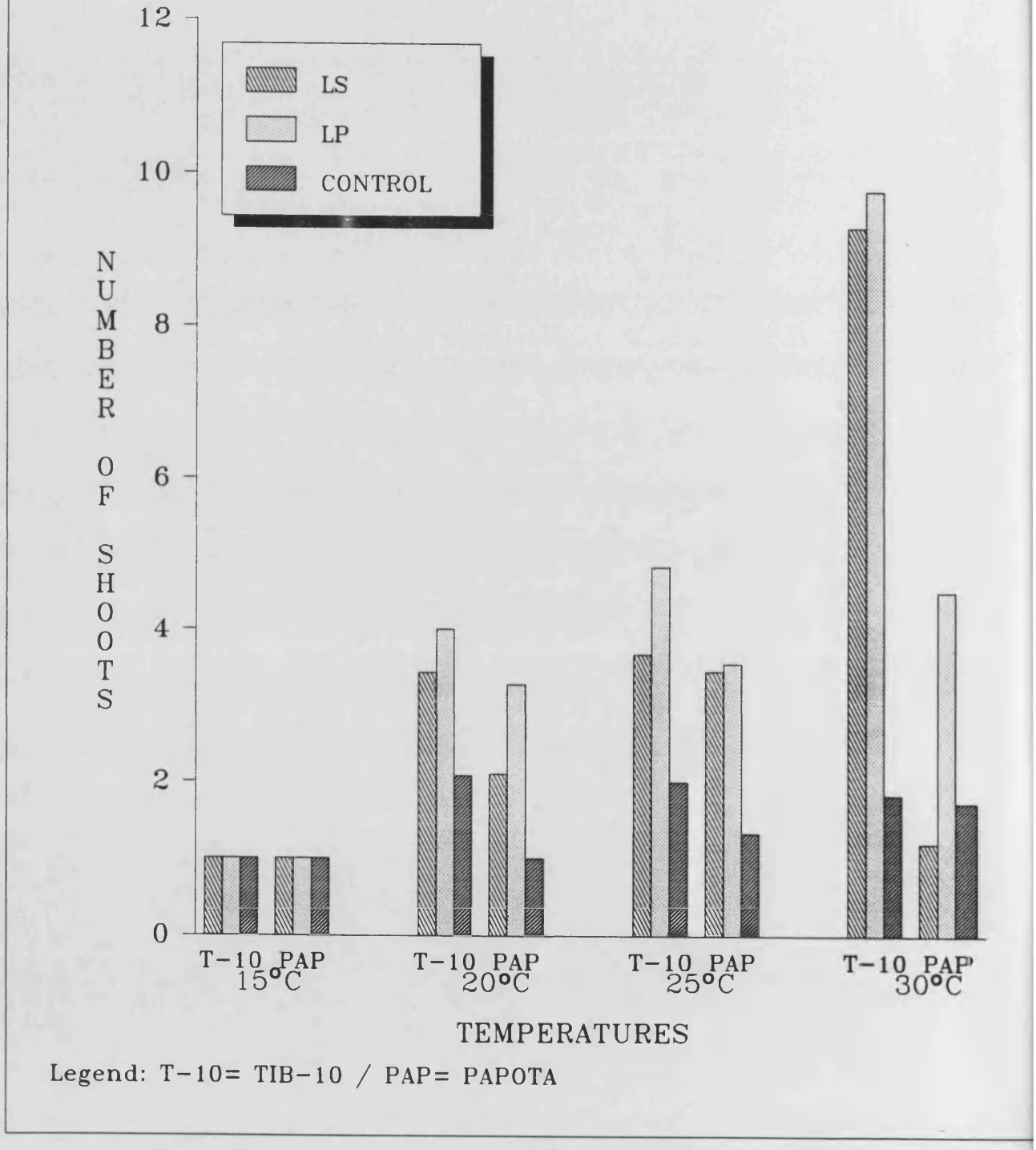
Basal medium: MII-t

Procedure: Culture of the explants under LP or LS overlay for 8 months

Temperatures: 15^0C, 20^0C, 25^0C and 30^0C

Light conditions: 16 hour photoperiod

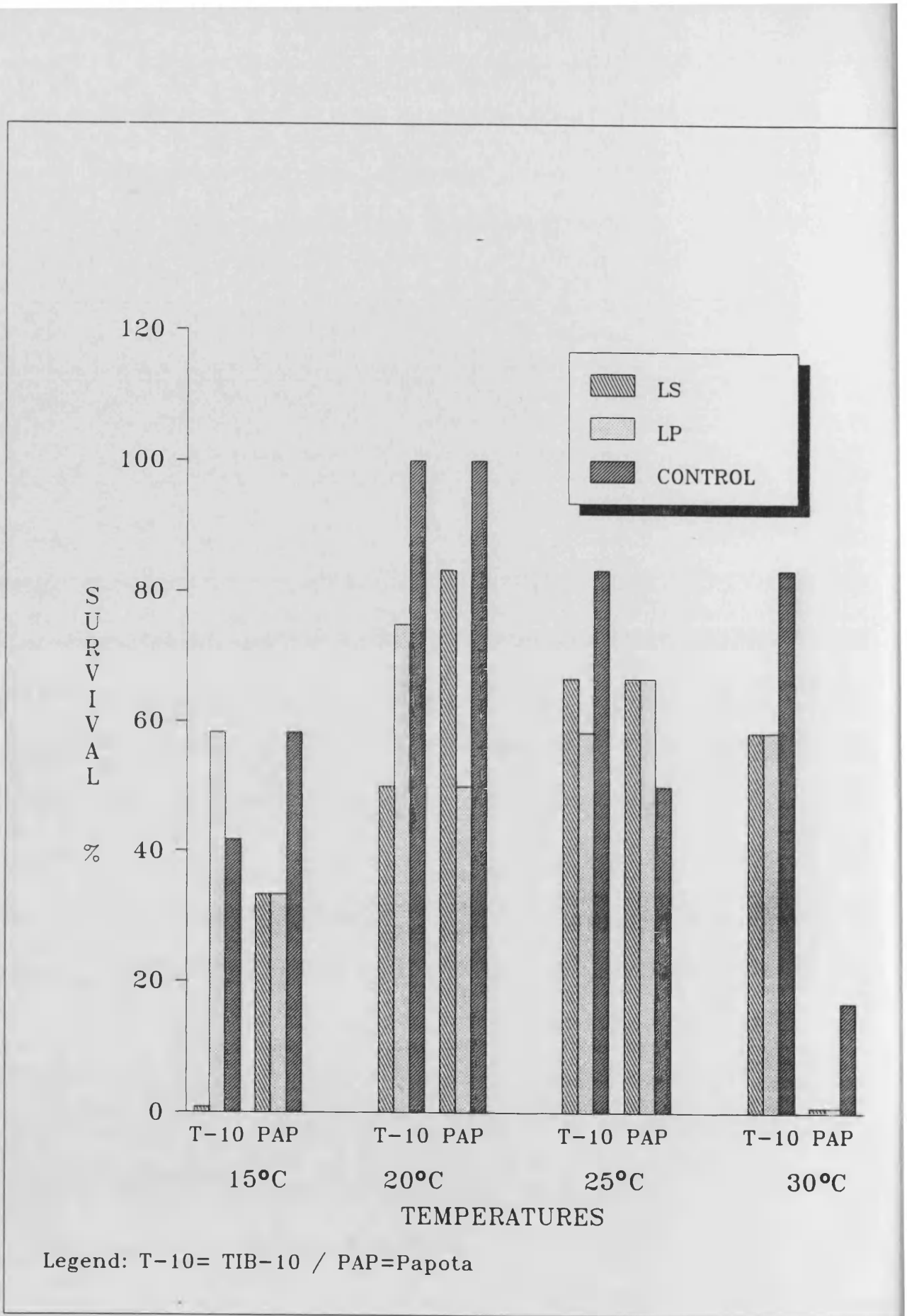
30 $\mu Mm^{-2}s^{-1}$PAR



Legend: T-10= TIB-10 / PAP= PAPOTA

Figure 3.1.12 Effects of four different temperatures on the production of shoots from sweet potato genotypes stored for 8 months under LP and LS overlay

Key: See details in Figure 3.1.11



Legend: T-10= TIB-10 / PAP=Papota

Figure 3.1.13 Effects of four different temperatures on the survival sweet potato genotypes stored for 8 months under LP and LS overlay

Key: See details in Figure 3.1.11

3.1.4 Discussion

The results from experiments on oil-overlay storage showed that, despite the genotype specificity, a reduction in growth rate of sweet potato cultures can be induced for germplasm storage purposes by the use of this technique with either LP or LS. Generally, LP proved to be more effective not only from the point of view of survival, but also producing plantlets with more axillary shoots which could be directly sub-cultured, thus increasing the possibilities of survival after storage. LS treated explants, on the other hand, showed better and faster signs of recovery after the storage.

Small explants (0.3-0.9 cm) performed very well and proved to be very suitable for oil-overlay conservation. Their use made the procedure easier and less time consuming because both the sub-culture and the oil treatment were carried out at the same time, thus avoiding the excessive manipulation of cultures and the risk of contamination. Generally the use of the oil-overlay induced the explants to produce more than one shoot per plant. The small explants were stimulated to produce more axillary shoots per explant under oil, particularly under LP, thus providing a better basis for survival. This enhanced production of non-adventitious shoots from the cultures developed from the small explants was probably a result of their axillary buds being produced almost entirely under the submerged conditions; with the longer explants, the axillary buds

already produced under more normal atmospheric conditions before being submerged, perhaps, would not respond to the new morphogenic stimuli. The extent of this effect was genotype specific: TIB-10 was more affected, producing a larger number of non-adventitious shoots per plant than Papota or CN-1367-2; with the latter fewer of the explants produced the larger number of non-adventitious shoots. CN-1367-2 proved to be very suitable for the oil-overlay storage as its development was very slow, however this genotype has a slower growth rate than others under normal maintenance conditions (see section 2.1.2).

The results from the second experiment showed almost no difference in survival between explants with or without the initial growth phase under both oil treatments, but the number of axillary shoots developed under LP was greater following the initial growth phase; there was no such difference under LS. However, as the data were collected after 10 months of culture when the axillary shoots were still being developed, it is possible that these results would eventually change with time.

The ideal temperature for conserving sweet potato cultures seems to be around 20^0C. At this temperature cultures from both Papota and TIB-10 under any treatment looked very healthy. These indications are similar to the results obtained by Allan (1979) who recommends 22^0C for the conservation of sweet potato.

All cultures stored under the oil-overlay either produced reduced leaves, which lost their original shape, becoming elliptical and elongated or they were without leaves, showing only the primordia.

Bridgen and Staby (1981) suggested that *Nicotiana tabacum and Chrysanthemum x morifolia* growing at low oxygen level and low pressure had their growth rates reduced by no other factor than the low concentration of oxygen. Also Moriguchi and colleagues (1988) suggested that callus of grape had their growth depressed when maintained under LS at 10^0C because the association of low temperature and low oxygen concentration.

The low concentrations of oxygen are probably responsible not only for the reduction of growth but also for the changes of shape and lack of development of leaves of explants preserved under oil. However other factors like the concentration of ethylene and CO_2 can not be discarded.

The lag phase observed during the recovery of nodal segments originated from either LP or LS treated cultures was also noticed by Mathur and colleagues (1991) during the regeneration of cultures of various species stored

under LP, the development of the referred cultures occurred after 60 days.

3.2 CRYOPRESERVATION

3.2.1 Introduction

The earliest reports indicating the possibility of survival of plant material after being subjected to ultra low temperatures date from 1956 (see reviews by Kartha, 1985 and Withers, 1987). In the last twenty years the freezing of plant organs and cells has become an accepted technique for their conservation, together with the widespread use of the plant tissue culture techniques. During this period there has been considerable progress in both fields. At present, various types of plant material including meristems and shoot-tips, cultured cells, callus, protoplasts, pollen and anthers, zygotic and somatic embryos and whole seeds of a number of species have been successfully recovered after freeze-storage at liquid nitrogen (LN) temperature for periods of time ranging from a few hours to years. Despite some relative successes there are no viable freezing methods by which long-term conservation of vegetatively propagated plant germplasm can be ensured; the methods are still in their experimental stage (see reviews by Kartha, 1985 and Withers, 1987).

Since the storage of explants in liquid nitrogen reduces the physical and chemical activities of the cells to negligible levels, cryopreservation is in theory, the best method to maintain germplasm because such conditions provide the greatest security against genetic variation in the long-term, except for the minor risks of mutation caused by the background radiation (Morris, 1980; Henshaw, 1982; Kartha, 1985).

Only a few biological materials can tolerate sub-freezing temperatures in their natural state without protection by a suitable chemical compound cryoprotectant or hardening. The mechanisms of cryoprotection are complex and not fully understood but the cryoprotectant compounds lower the temperatures at which freezing first occurs and change the size of ice crystals. Their colligative properties minimise the action of increased electrolyte concentration resulting from the conversion of water into ice (Nash, 1966). High solubility and low toxicity are among the essential characteristics of successful cryoprotectants.

Glycerol was the first chemical to be used as a cryoprotectant (Polge *et al.*, 1949), followed by dimethyl sulfoxide (DMSO) (Lovelock and Bishop, 1959). At present there are many other compounds being used as cryoprotectants but DMSO and glycerol are still the most commonly used in the search for cryopreservation, generally in concentrations around 5-10% and 10-20% (v/v), respectively (Morris, 1980; Kartha, 1987).

Most of the cryoprotectants have some toxicity at higher concentrations. When cryoprotectants at non-toxic levels do not afford enough protection against cryo-injuries, methods using mixtures of compounds, or pre-culturing the explants in medium with osmotically active compounds such as sucrose, mannitol, proline and sorbitol, or cold hardening can be beneficial (Frinkle *et al.*, 1985; Kartha, 1987).

The freezing procedures can involve either fast or slow rates of cooling. Ultra-fast freezing was used with success by Grout and Henshaw (1978), for the cryopreservation of potato shoot-tips, the explants were placed on a hypodermic needle and plunged directly from room temperature into LN. The greatest advantage of ultra fast freezing is the simplicity of the technique, but it does not seem to be suitable for a wide range of species.

Slow freezing has been successfully used with a wider range of species and types of explants. The method usually has two well defined stages: the first involves a slow-cooling procedure to reach a critical temperature which is variable according to the characteristics of the explant, but usually in the vicinity of $-30^{0}C$ and the second stage involves rapid-freezing to $-196^{0}C$, normally achieved by plunging the explants directly into LN. The most common cooling rates used for the slow freezing are between 0.5 and $2^{0}C.min^{-1}$; at these rates the cells reach equilibrium with the external ice by efflux of water and they become shrunken, provided that the cell is sufficiently water permeable. Under such conditions the

formation of intracellular ice, which would be responsible for the cryo-injury, is reduced to a minimum (see reviews by Meryman *et al.*, 1977; Withers, 1980 and Kartha, 1985).

The storage of frozen explants should be carried out at a suitable low temperature to prevent ice recrystalization. The best temperatures are below $-120^{0}C$, but the most recommended is in a LN refrigerator in which the temperature of liquid is $-196^{0}C$ and the vapour is $-150^{0}C$ (Withers, 1987).

Thawing is as critical as all the previous operations, since at this stage there is a serious risk of recrystallization of ice. The warming is usually obtained by plunging the explants rapidly into a water bath or liquid medium either at $40^{0}C$ or at room temperature.

The procedure for recovery of explants varies according to the type of material but generally a special medium and controlled environmental conditions are required for each explant.

Sweet potato cells were one of the first types of plant material to be successfully cryopreserved. This was achieved by pre-conditioning the cells by culturing them for one week with a gradual increase of sucrose levels achieved by adding 1 ml of a 50% sucrose solution to 77 ml of the cultures at 24 hour intervals and finally slowly adding 2.5% glycerol and DMSO over a period of 60-90 minutes prior to the freezing (Latta, 1971). Although the cryopreservation of sweet potato cells was

successful, sweet potato is well-known as a difficult species to survive cryopreservation (IBPGR, 1988). Recently, Kadir and Rhodes (1989) were able to regenerate 50% of the shoot-tips frozen to -45°C cryoprotected with 10% DMSO, however no survival was obtained after deep freezing at -196°C.

The aim of the experiments on cryopreservation was to develop a method for the long-term storage of sweet potato germplasm with minimal damage to the genotypes. The tests were carried out in two stages: **a)** effects of toxicity of cryoprotectants and **b)** cryopreservation trials which were first based on the freezing of shoot meristems and the second on the freezing of root-tips.

Cryopreservation of the shoot meristems was unsuccessful, whereas that of the root-tips showed good results.

3.2.2 Material and Methods

3.2.2.1 Plant material

The experiments on cryopreservation were carried out at first with shoot-meristems and later with root-tips.

The shoot-meristems used to test both the toxicity of cryoprotectants and the freezing procedure were obtained from axillary buds of plants growing in the glass house at a temperature of 24-27°C and 16 hour photoperiod. The excision of meristems followed the procedure described in section 2.1.2.

The root-tips used for the experiments on the toxicity of cryoprotectants and cryopreservation were taken from 10-day old *in vitro* plantlets which had originated from nodal segments of stock shoot cultures. The root-tips used for tests on toxicity were 0.3-0.4 cm in length and 0.05-0.1 cm in diameter, the cryopreservation was carried out using 0.1-0.2 cm root-tips with 0.05-0.1 cm diameter. An initial culture stage was not used because at this stage the roots were actively growing, and with further growth the tips would have been too big for the freezing tests.

Brondal, CN-1367-2 and Papota were the main genotypes used for the experiments on toxicity , however only Brondal and Papota were used for cryopreservation because they reacted better to the cryoprotectants than CN-1367-2. Also the genotype TIB-10 was used for the tests on cryopreservation of root-tips.

3.2.2.2 Methods

3.2.2.2.1 Toxicity of cryoprotectants

The cryoprotectants tested for toxicity were DMSO, glycerol, mannitol, proline, PVP-40, sorbitol and sucrose. Generally the levels of cryoprotectant tested varied from 2.5 to 10% (v/v or w/v) (see section 3.2.3 for details).

Filter-sterilised DMSO and glycerol were added to a heat sterilised liquid MII-m (Table 2.1) nutrient

medium (complete composition for shoot-meristems and without the growth regulators for root-tips) and explants were immersed in the mixture for two hours at room temperature: after that they were washed with liquid MII-m and transferred to a semi-solid MII-m and cultured at 25±2°C with 16 h photoperiod. The other cryoprotectants were added to semi-solid MII-m before the sterilisation, the explants were directly cultured in the mixture of medium and cryoprotectants at the tested concentration. After three days of culture at 25±2°C with 16 hour photoperiod the explants were transferred to a fresh semi-solid MII-m without cryoprotectant, then allowed to grow for one month.

3.2.2.2.2 Cryopreservation

When a single cryoprotectant was used, the addition was made two hours before the freezing was carried out; however, when two or more cryoprotectants were used (for shoot meristems only), generally one of them was added to the MII-m as a pre-treatment for three days and the other was added two hours before freezing.

The technique used for cryopreservation was based on that developed by Henshaw and colleagues (1985). The samples were slow-cooled at a rate of 0.5°C.min^{-1} to various temperatures prior to their immersion in liquid nitrogen (LN) at -196°C.

Batches of twelve explants were lined up on a 2.2 x 0.3 cm lens tissue strip laid on an aluminium foil strip

of the same size and covered by a second lens tissue strip. Each of the carriers containing the explants were placed into 1.5 cm petri dishes with a 2 ml cryoprotectant solution in liquid MII-m (complete composition for shoot-meristems and without the growth regulators for root-tips). After two hours, each of the carriers was inserted into a 1.8 ml cryotube with a drop of the cryoprotectant solution. The cryotube was then plunged into a $-5^{0}C$ methanol bath controlled by a programmable freezer ETP-3 Exatol (Neslab) and allowed to stabilise for 10 minutes. Ice formation was then induced in the cryoprotectant solution by touching the base of the cryotube with the tip of a pair of liquid nitrogen-cooled forceps.

After a further 10 minutes the reduction of temperature to $-30^{0}C$ was started at the rate of $0.3^{0}C.min^{-1}$ and two cryotubes were withdrawn from the freezer at $5^{0}C$ intervals. The carriers were then removed from those cryotubes; one was thawed and the survival was checked, the other was plunged into LN for 20 minutes in order to complete the last stage of the freezing process.

Thawing was carried out by quickly placing the carriers into small petri dishes containing 15 ml liquid MII-m (as above) at room temperature, where they were maintained for 30 minutes. This method enabled thawing and washing at the same time.

While the survival rates of shoot meristems were verified by sub-culture onto semi-solid MII-m, the root-

tips had their viability checked by adding two drops of a fluorescein diacetate (FDA) solution in acetone (Widholm, 1972) and observing the activity under an Olympus BHT epifluorescence microscope with 400-490 nm excitation filter, 500-515 nm mirror and 530 nm barrier filter.

3.2.3 Results

The experiments on cryopreservation of sweet potato explants followed two stages: a) the analysis of the toxic effects of cryoprotectants on the explants, b) the cryopreservation itself.

3.2.3.1 Effects of the cryoprotectants on the survival and development of the cultures

Before the freezing experiments were carried out various tests were made in order to check the toxic effects which the cryoprotectants would have on the explants. This was an important step because any damaging effect on the explants, other than the ones caused by the cryopreservation should be avoided, otherwise the effects of freezing could be disguised by the toxicity of the cryoprotectant.

Generally the toxicity studies were based on the survival of explants two weeks after subcultured (see section 3.2.2.2.1 for details), when the survival

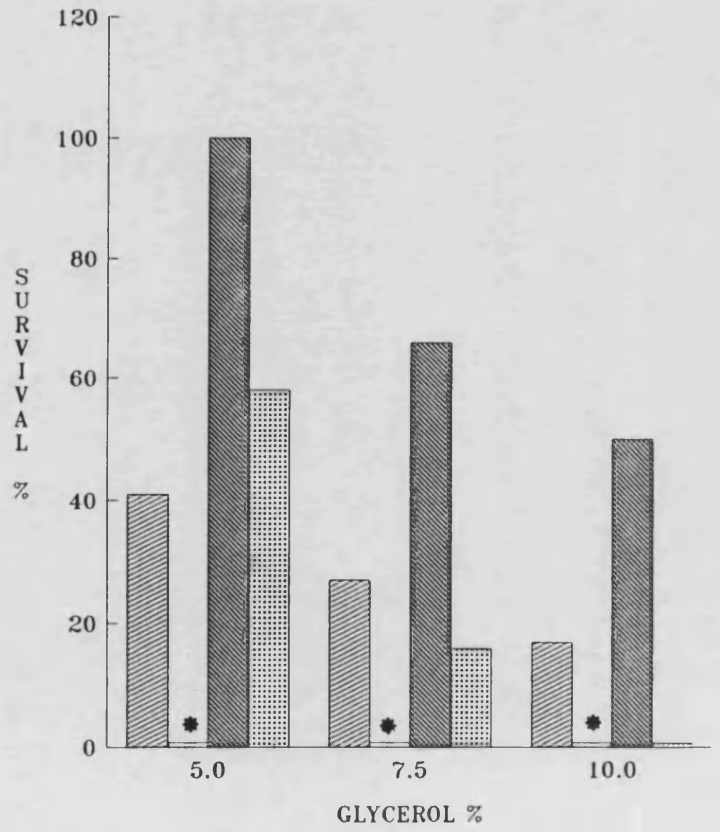
rates were high other factors such as callus formation and abnormal development were also observed.

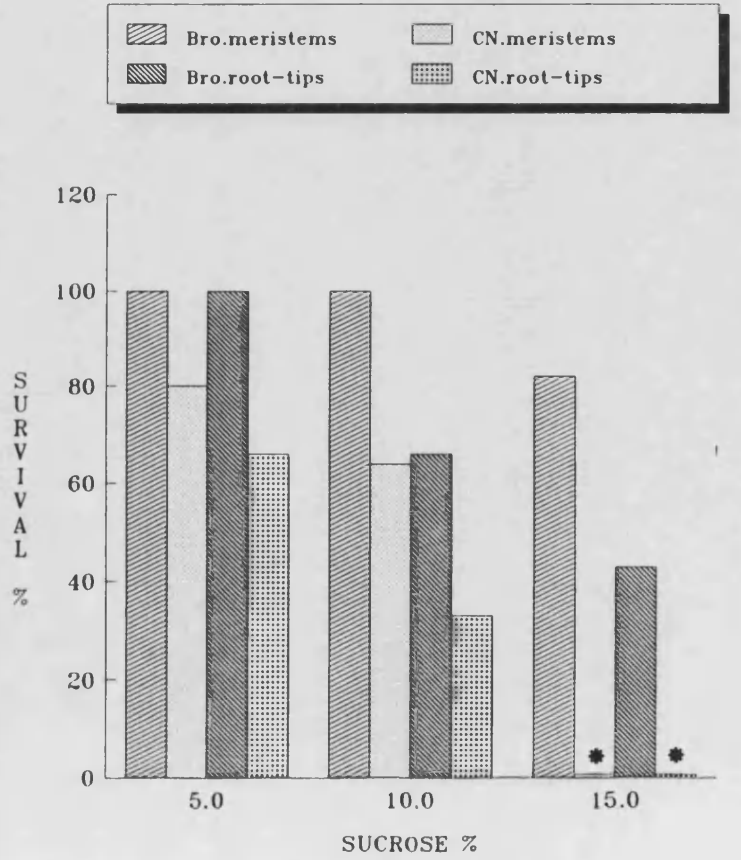
The reactions of explants to the cryoprotectants were generally similar but they varied in intensity according to the genotype and organ used (Figures 3.2.1 to 3.2.5).

Usually, the survival of explants was inversely proportional to the concentration of cryoprotectant, but root-tips were less affected by the toxicity of cryoprotectants than shoot meristems; these effects are shown in figures 3.2.1, 3.2.2, 3.2.4 and 3.2.5. PVP-40 was one of the exceptions to that pattern and shoot meristems and root-tips of Brondal and CN-1367-2 which were treated with PVP-40 alone or with glycerol in a two step treatment (5% PVP-40 and 5% glycerol (w/v) showed good survival at all levels tested (Figures 3.2.2 and 3.2.8), however when other aspects were considered it could not be recommended as a cryoprotectant for these organs because it induced callus formation on root-tips and shoot meristems (Figure 3.2.3) and multiple shoots on meristems, which may be of adventitious origin. Treatment involving glycerol (5 or 10%, v/v) with sorbitol (2.5 or 5%, w/v) treatment was also an exception to that pattern; figure 3.2.5 shows very high levels of survival of shoot meristems of Brondal and CN-1367-2 after such treatment. The use of mannitol (three-day pre-treatment) with glycerol (two hours prior to freezing) killed the shoot meristems of Brondal and CN-1367-2.

DMSO which is one of the better known cryoprotectants was quite toxic to shoot meristems at higher concentrations (above 7.5%) either alone or mixed with glycerol, but it was almost non-toxic to root-tips even at 15% (v/v) (Figures 3.2.4; 3.2.5); no callus was induced by DMSO. When meristems of Brondal and CN-1367-2 were treated with a mixture of DMSO and glycerol each at 2.5% (v/v) and sucrose at 8%, 10% and 12% (v/w), despite some callus formation the effects on the survival were very good (see the controls of Figure 3.2.7). Higher levels of sucrose (15%, 20% and 25%) were used for shoot meristems of genotype Papota obtained from *in vitro* and *in vivo* donor plants (see Figure 3.2.6), the survival was good considering the high levels of sucrose, but the explants did not develop at 20% and 25%; meristems originated from plants growing in the green house proved to be better for this type of tests than meristems obtained from *in vitro* cultures.

The genotype CN-1367-2 was more affected by the toxic effects of the cryoprotectants tested than genotypes Brondal and Papota.





Legend: Bro.= Brondal CN.= CN-1367-2
* not tested

Figure 3.2.1 Effects of various concentrations of sucrose and glycerol on the survival of CN-1367-2 and Erondal genotypes

Key:

Number of replicates: 12

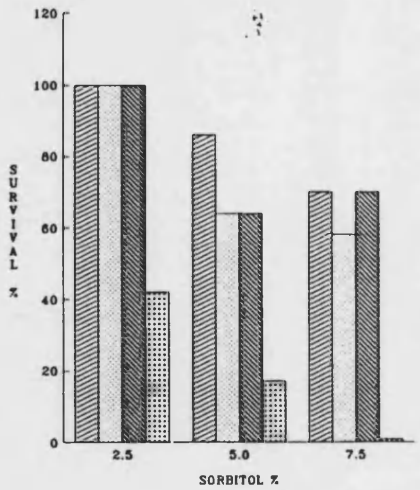
Basal media: MII-m

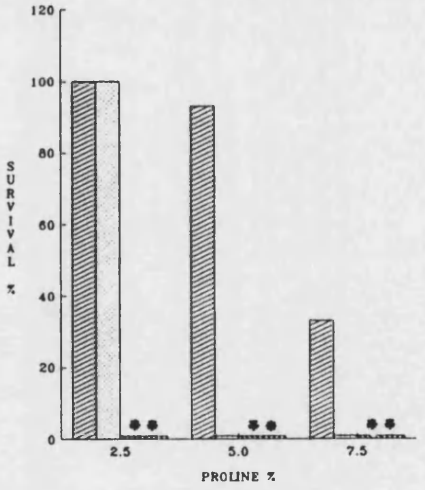
Procedure: Three days growth on semi-solid medium supplemented with the cryoprotectant at tested concentration followed by subculture on basal medium without cryoprotectant. Survival was based on growth of explants

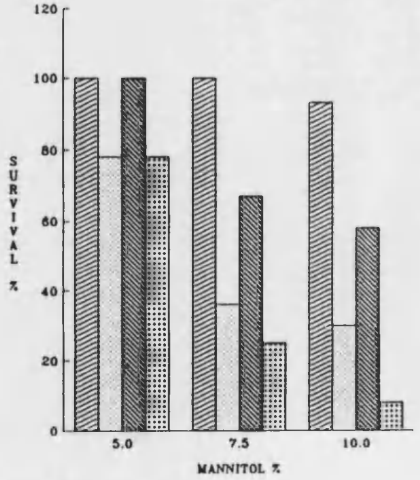
Culture conditions: Temperature: $25\pm2^{0}C$

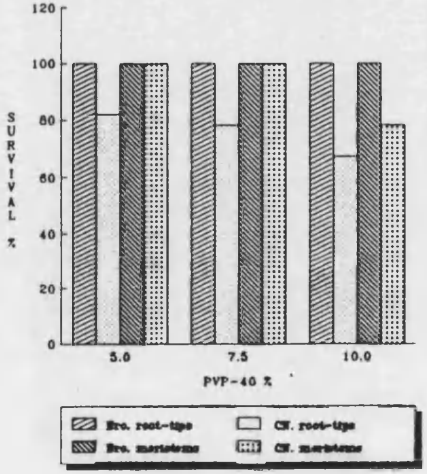
Light: 16 hour photoperiod

30 $\mu Mm^{-2}s^{-1}PAR$





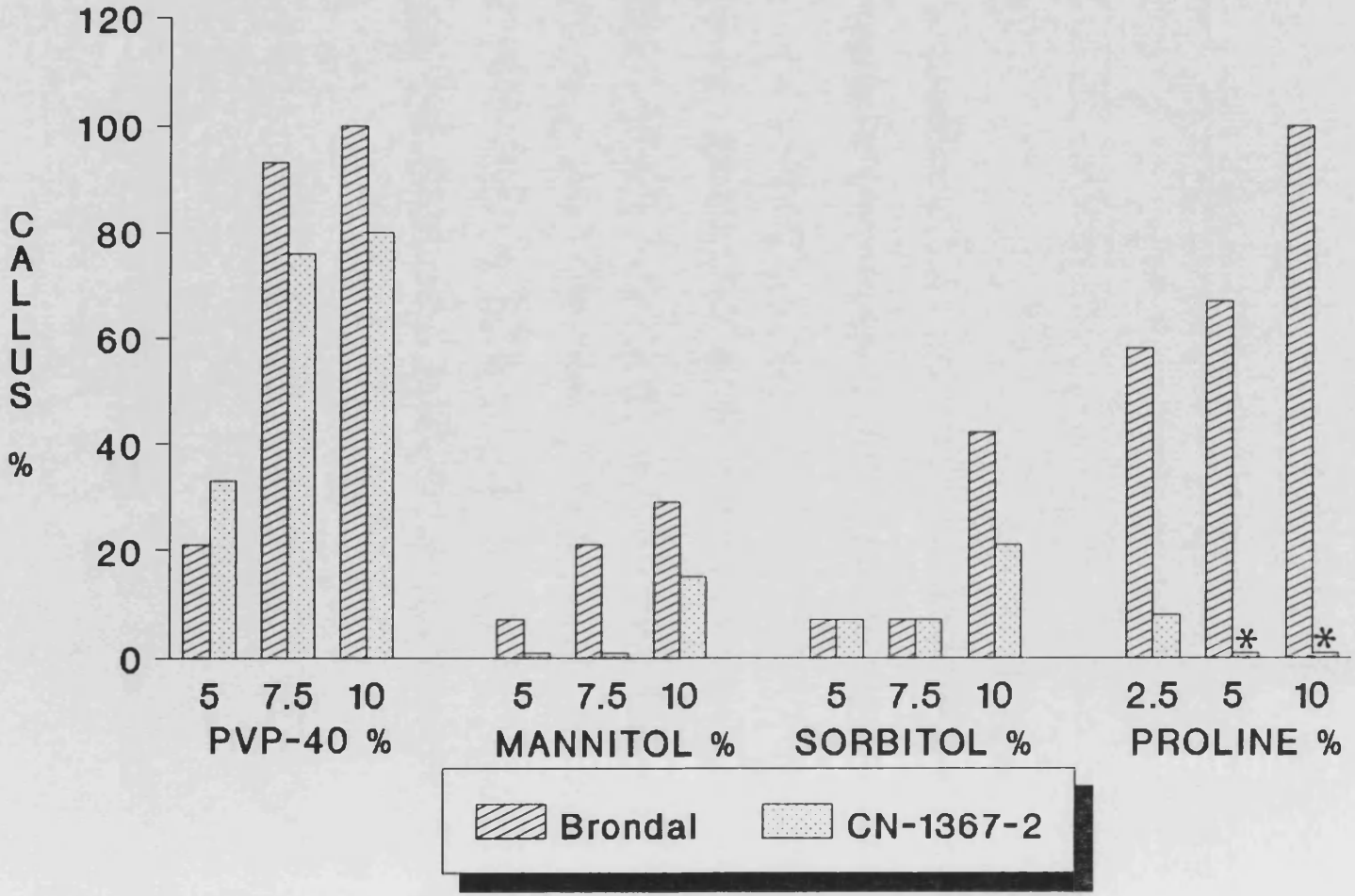




Legend: Bro. = Brandal CN =CN-1967-2
* not tested

Figure 3.2.2 Effects of various concentrations of proline, PVP-40, mannitol and sorbitol on the survival of CN-1367-2 and Brondal genotypes

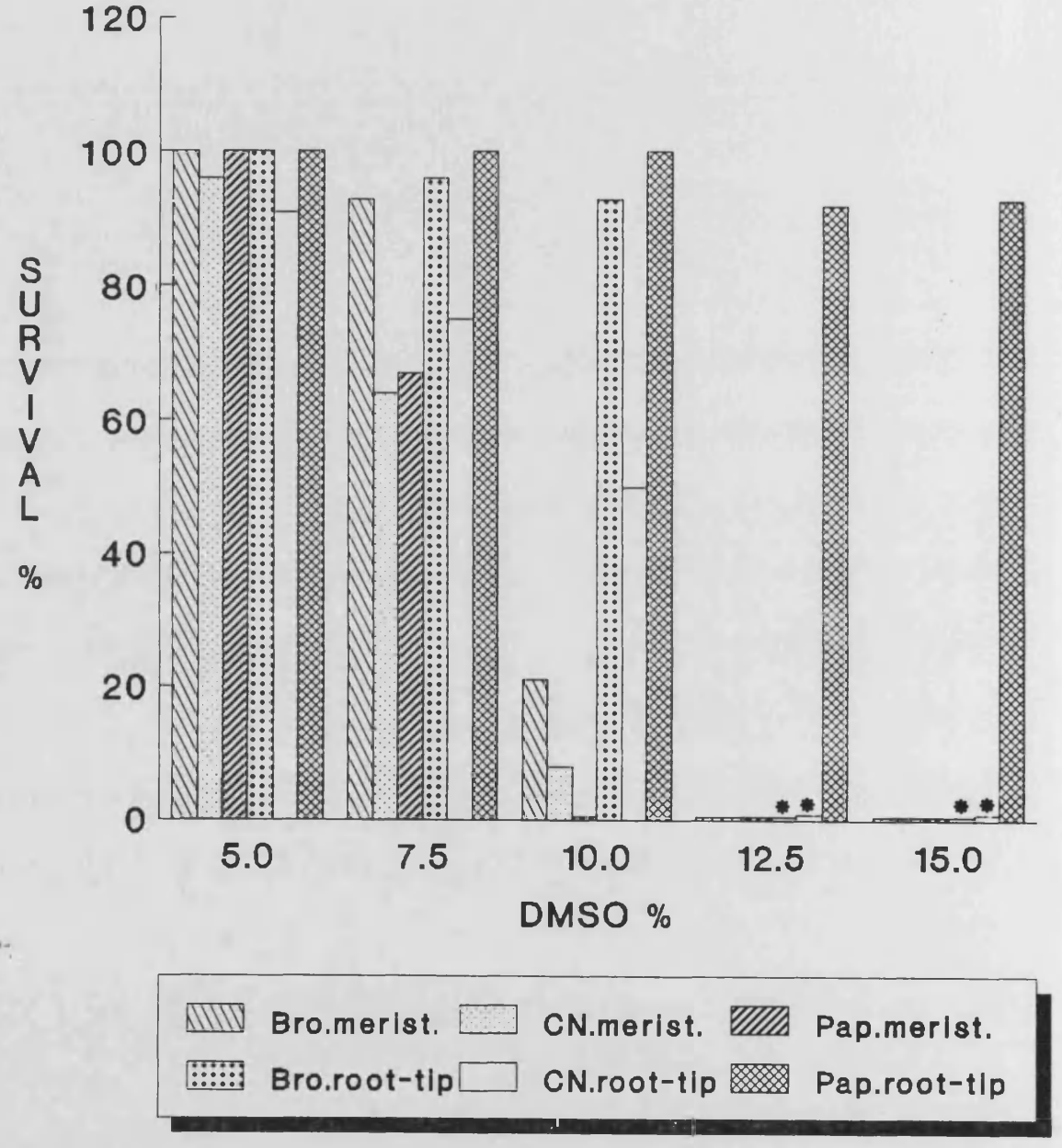
Key: See details in Figure 3.2.1



LEGEND: *not tested

Figure 3.2.3 Effects of various concentrations of proline, PVP-40, mannitol and sorbitol on the callus formation of CN-1367-2 and Brondal shoot meristems cultures

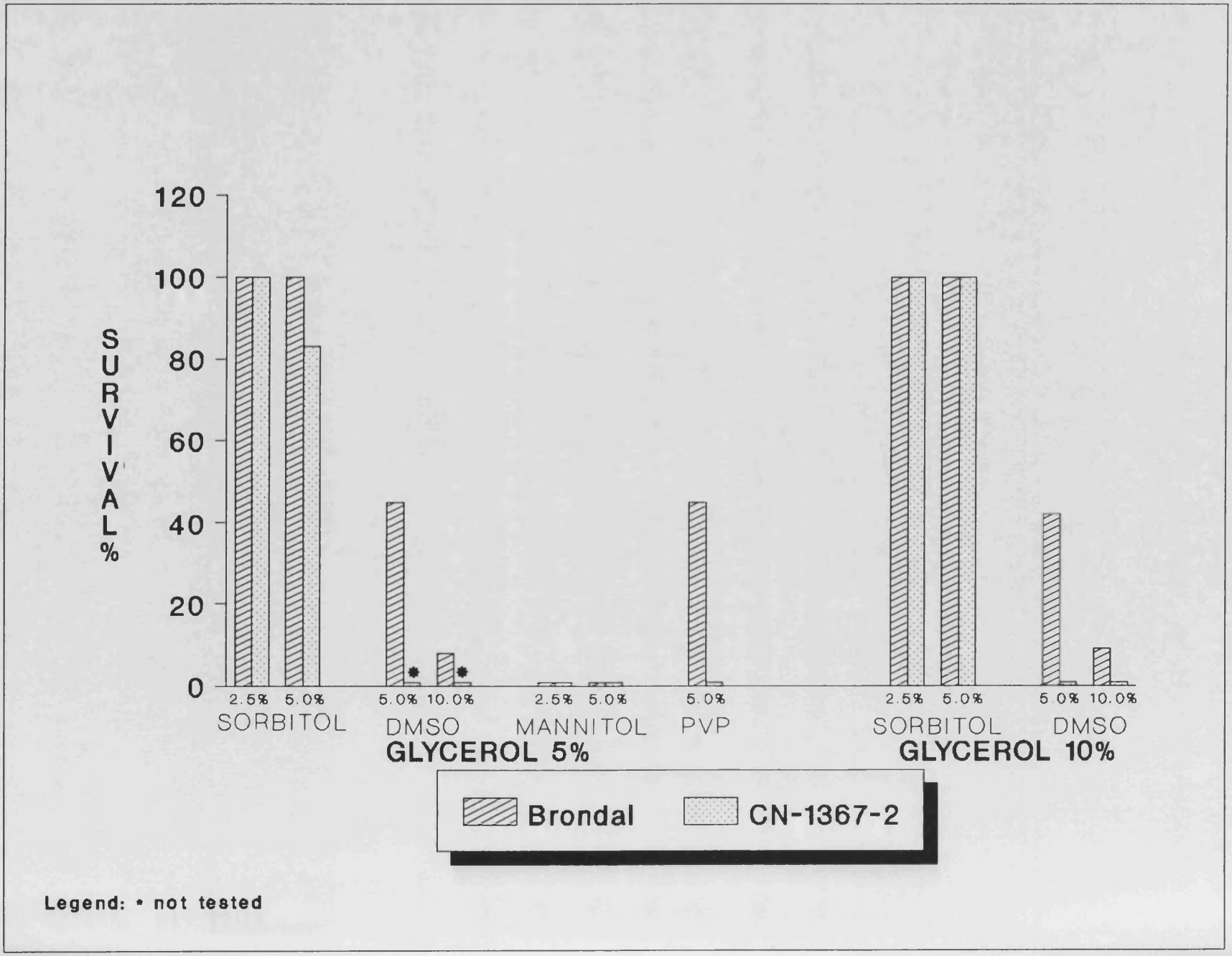
Key: See details in Figure 3.2.1



Legend: Bro- Brondal CN- CN-1367-2
Pap.- Papota * not tested

Figure 3.2.4 Effects of various concentrations of DMSO on the survival of CN-1367-2, Brondal and Papota genotypes

Key: See details in Figure 3.2.1



Legend: * not tested

Figure 3.2.5 Effects of mixtures of glycerol to sorbitol, DMSO and PVP-40 on the survival of CN-1367-2 and Brondal shoot meristems

Key: See details in Figure 3.2.1

3.2.3.2 Cryopreservation

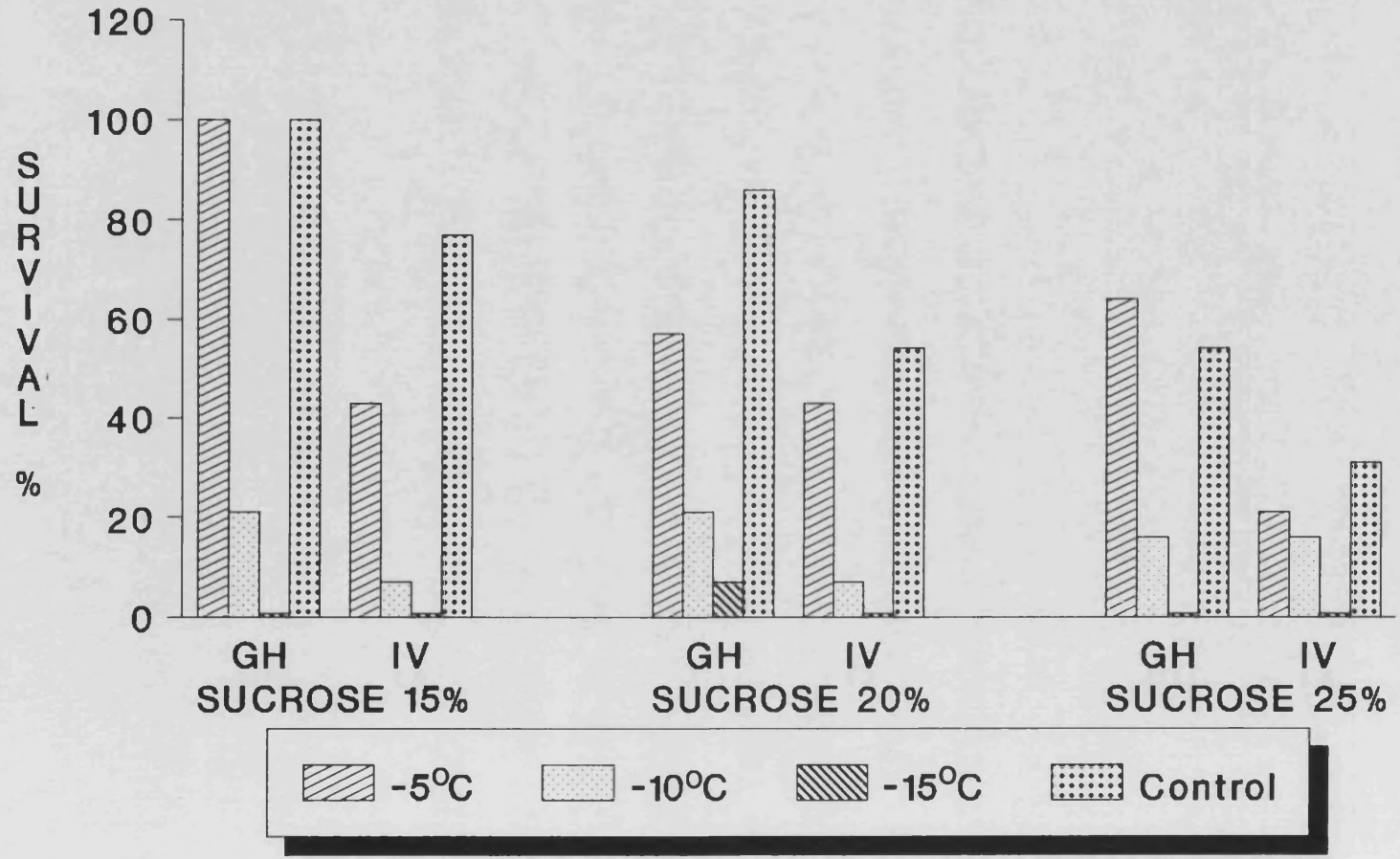
3.2.3.2.1 Cryopreservation of shoot meristems

Many tests were made on the cryopreservation of sweet potato shoot meristems using the cryoprotectants which were tested previously, but generally, cryoprotectants which had been recommended as good for survival were not effective against freeze damage and the explants were dead when temperature reached -10°C, with the explants being killed at temperatures between -5°C and -10°C.

Unfortunately, it was not always possible to use here the genotypes which had shown a better response to the toxicity of the cryoprotectants during the previous experiments because of their restricted availability during periods of red mite infestation in the green house (see section 2.1.1).

The best results from the cryopreservation of shoot meristems can be seen in the Figures 3.2.6, 3.2.7 and 3.2.8. The shoot-meristems of Papota showed 9% survival at -15°C, when treated with a mixture of DMSO and glycerol each at 2.5% (v/v) and sucrose at 20% and also showed 20% survival at -10°C when the same mixture had the sucrose concentration varying from 15%, 20% and 25% (see Figure 3.2.6); but when the same mixture with lower levels of sucrose (8%, 10% and 12%) was tested with genotypes Brondal and CN-1367-2 the shoot meristems were dead at temperatures below -5°C (see Figure 3.2.7).

The genotype Papota showed a good survival (above 80%) at -10°C when the shoot meristems were treated with glycerol (5% and 10% in a tree-day pre-treatment) with sorbitol (2.5% and 5% in a two hours treatment), but the treatment was not effective when temperature was decreased to -15°C (Figure 3.2.8 and Plate 3.2.1).



Legend:
GH- explants from in vivo donor plants
IV- explants from in vitro donor plants

Figure 3.2.6 Survival of Papota shoot meristems after cooled to -15^{0}C using a mixture of 2.5% DMSO and 2.5% glycerol with three levels of sucrose as cryoprotectants. These shoot-meristems were originated from plants growing in the glass house (GH) and from *in vitro* cultures (IV).

Key:

Number of replicates: 12

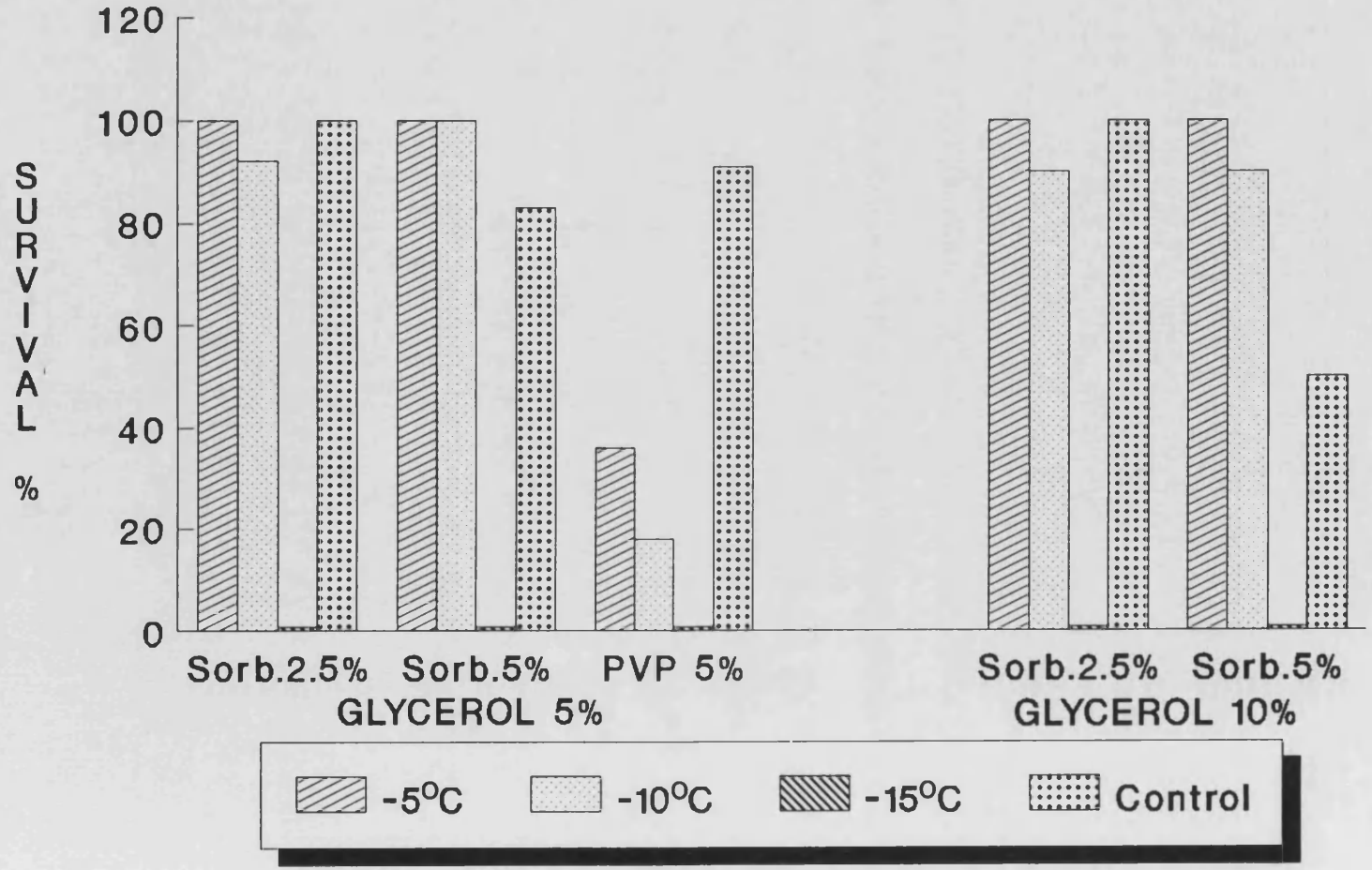
Procedure:
1) 3 days pre-culture in MII-m supplemented with sucrose at the indicated concentrations
2) 2 hours in liquid MII-m supplemented with 2.5% DMSO and 2.5% glycerol
3) Slow freezing to -15^{0}C at 0.3^{0}C.min^{-1}
4) Thawing and washing in liquid MII-m
5) Sub-culture in semi-solid MII-m reducing by 5% the sucrose level every 3 days.

Culture conditions: Temperature: 25±2^{0}C
Light: 16 hour photoperiod
30 μMm^{-2}s^{-1}PAR


Brondal
CN-1367-2
120
100
80
60
40
20
0
SURVIVAL %
-5
-10
-15
Contr.
-5
-10
-15
Contr.
-5
-10
-15
Contr.
TEMPERATURE °C
SUCROSE 8%
SUCROSE 10%
SUCROSE 12%

Figure 3.2.7 Survival of genotypes Brondal and CN-1367-2 shoot meristems after cooled using a mixture of 2.5% DMSO and 2.5% glycerol with three levels of sucrose as cryoprotectants.

Key: See details in Figure 3.2.6



Legend: Sorb.- sorbitol
PVP- PVP-40

Figure 3.2.8 Survival of Papota shoot meristems after cryopreservation using mixtures of cryoprotectants

Key:

Number of replicates: 12

Procedure: 1) 3 days pre-culture in MII-m supplemented with sorbitol or PVP-40 at the indicated concentrations

2) 2 hours in liquid MII-m added by 5 or 10% (v/v) glycerol

3) Slow freezing to $-15^{0}C$ at $0.3^{0}C.min^{-1}$

4) Thawing and washing in liquid MII-m at room temperature

5) Sub-culture on semi-solid MII-m

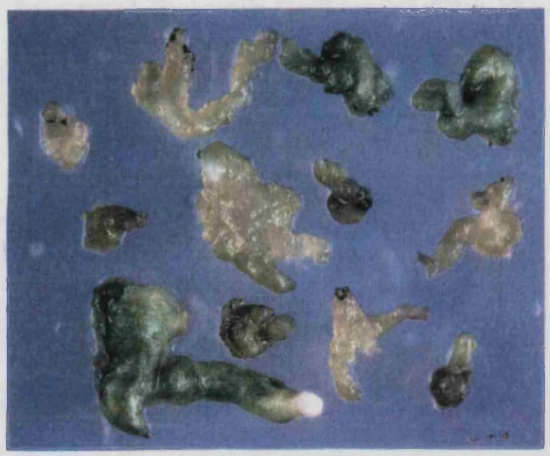
Culture conditions: Temperature: $25\pm2^{0}C$

Light: 16 hour photoperiod

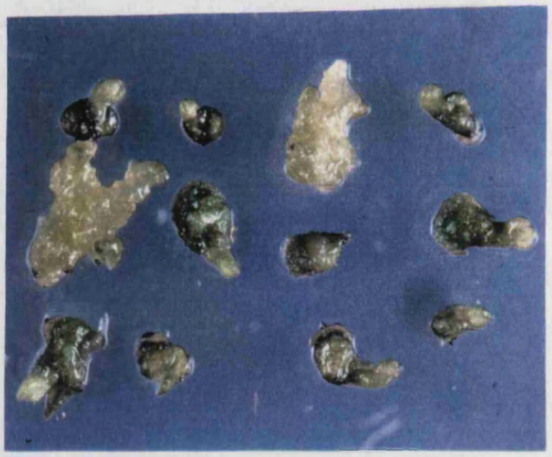
30 $\mu Mm^{-2}s^{-1}PAR$

Plate 3.2.1

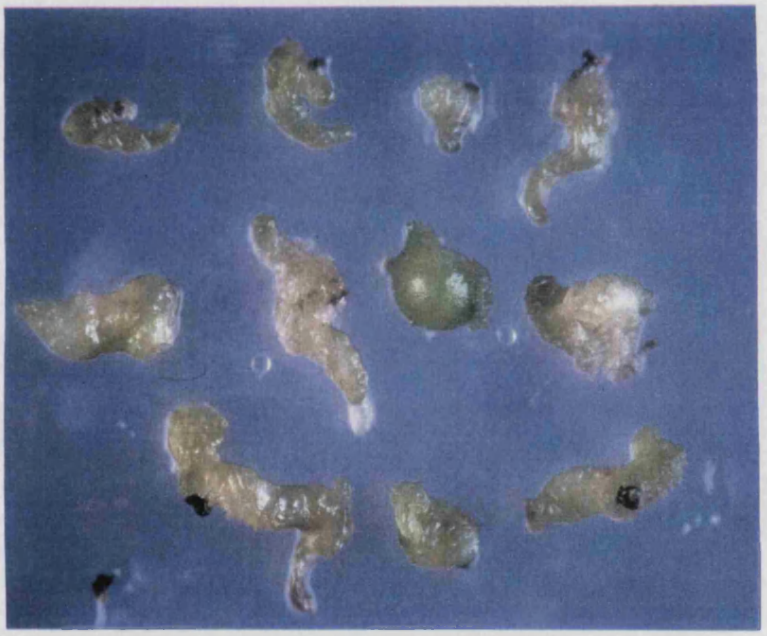
a



b



c



d

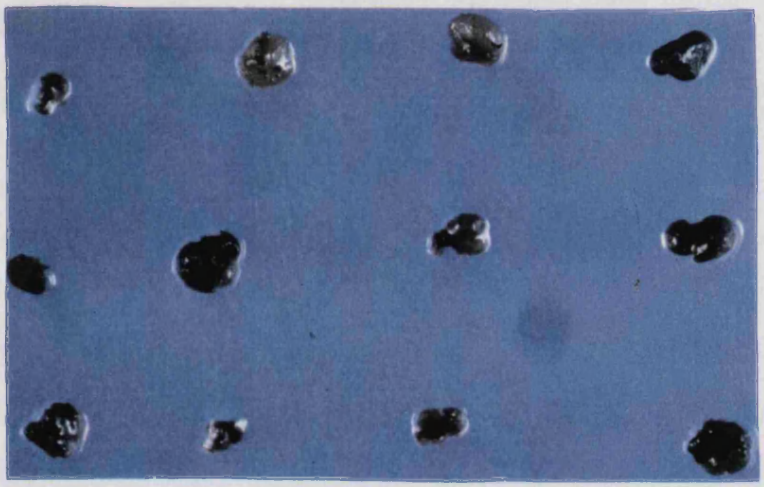


Plate 3.2.1 Regeneration of genotype Papota meristems after been frozen using 5% (w/v) sorbitol in a three day pre-treatment and 5% (v/v) glycerol two hours treatment.

a) Frozen to -10°C (x 1.35).

b) Frozen to -5°C (x 1.35).

c) Control at 25°C (x 2.2)

d) Frozen to -15°C (x 3.6)

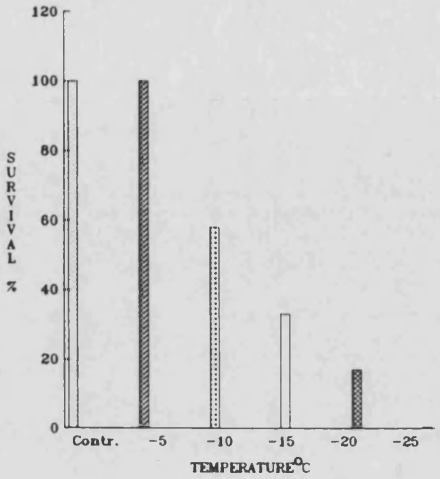
3.2.3.2.2 Cryopreservation of root-tips

Three successful tests were carried out with root-tips and all of these tests used 10% (v/v) DMSO as cryoprotectant.

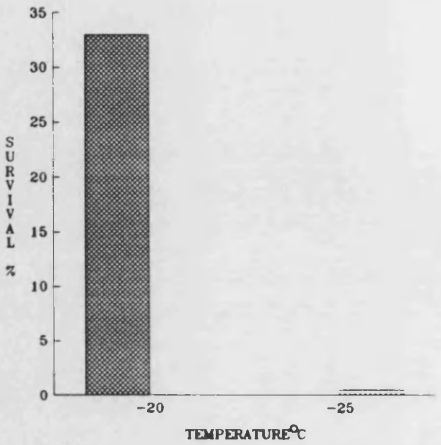
A previous small test on the cryopreservation of root-tips protected with 10% (v/v) DMSO showed some evidence that under these conditions the root-tips could survive until a temperature of -20°C was reached, although there was no survival when the explants were subsequently transferred to LN (-196°C). An alternative procedure to overcome this problem was to maintain the root-tips at -20°C for 10 minutes to allow for stabilisation of the system before transfer to LN; the results showed survival of explants not only after the 10 minutes at -20°C but also at -196°C (<

Figures 3.2.9, 3.2.10 and 3.2.11).

SLOW FREEZING



STABILIZATION FOR 20 MINUTES



FREEZING AFTER STABILIZATION

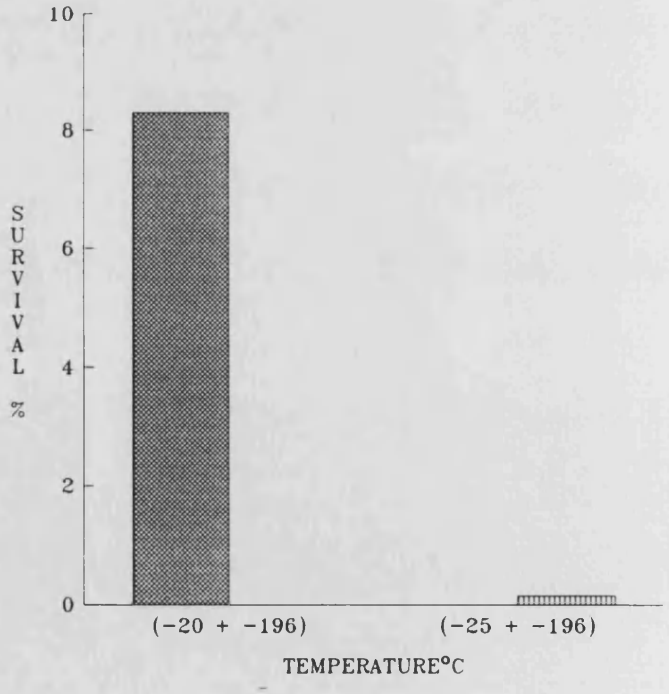


Figure 3.2.9 Survival of Brondal root-tips after cryopreservation using 10% DMSO as cryoprotectant. Survival was based on FDA.

Key:

Number of replicates: 14

Procedure:

1) 2 hour treatment with 10% (v/v) DMSO in liquid MII-m (without growth regulators) at room temperature.

2) Slow freezing to $-20^{0}C$ at $0.3^{0}C.min^{-1}$

3) 10 minutes at $-20^{0}C$

4) Deep freeze in LN at $-196^{0}C$

5) Thawing and washing in liquid MII-m at room temperature

6) Staining with FDA for microscope observations

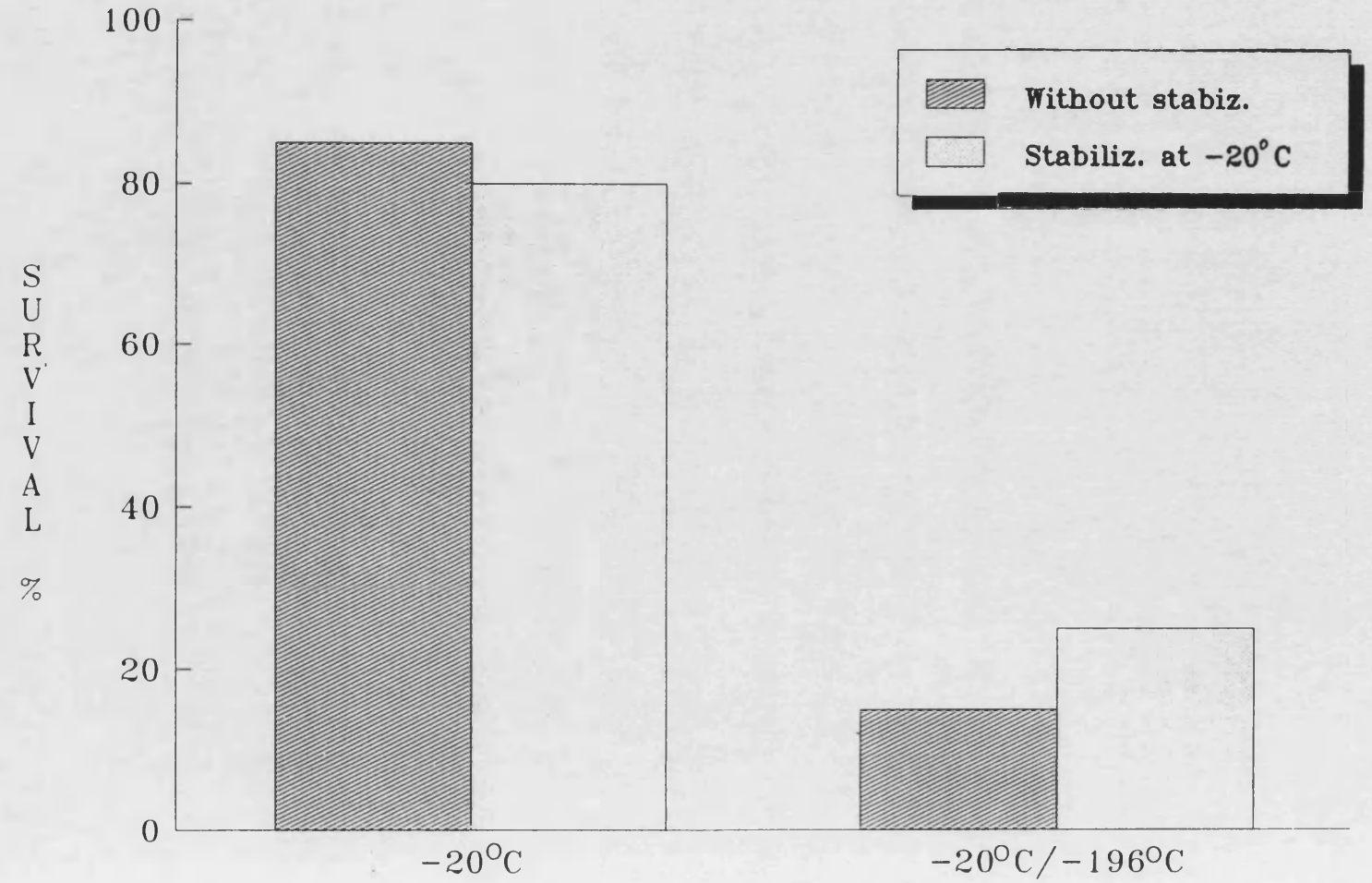

100
80
60
40
20
0
SURVIVAL %
Without stabiz.
Stabiliz. at −20°C
−20°C
−20°C/−196°C

Figure 3.2.10 Survival of Papota root-tips after cryopreservation using 10% DMSO as cryoprotectant. Survival was based on FDA.

Key:

Number of replicates: 20

Procedure: the same as Figure 3.2.9

3.2.4 Discussion

Sweet potato is a tropical crop which suffers damage when the temperature drops below 10^0C (Kassan, 1976). Although it is recognised as a recalcitrant species with regards to cryopreservation (IBPGR, 1988), the results obtained in these experiments showed that it is possible to obtain viable sweet potato root-tips after freezing to -196^0C.

Most of the successful reports on the cryopreservation of plant species have been based on the use of DMSO as a cryoprotectant (Kartha, 1987; Morris, 1980). However with sweet potato DMSO proved to be very toxic to shoot meristems at levels which might protect the cells against cryo-injury (above 7.5%), but at 5% and 7.5% (v/v) it showed acceptable toxicity though shoot meristems did not survive the freezing. The fact that the root-tips were less affected by DMSO than shoot meristems was probably one of the most important factors in their successful cryopreservation.

These are not the first success in the freezing of root-tips. Bajaj (1987) also obtained some survival on the cryopreservation of 0.5-1 cm potato root-tips in a solution containing 5% (v/v) DMSO, 5% (w/v) sucrose and 5% (v/v) glycerol; the tips remained white, showed some elongation and a mass of thin roots was formed after a lag period of 2-5 weeks, although no further development is reported.

Recently, Benson and Hamill (1991) obtained 20% survival on the cryopreservation of transformed hairy roots of *Beta vulgaris* and *Nicotiana rustica*.

The results obtained here on the cryopreservation of root-tips can also be a useful tool in the studies of transformation of sweet potato.

CHAPTER 4

INDUCTION OF SHOOT FORMATION ON ROOT CULTURES

4.1 Introduction

Isolated root cultures have provided experimental systems for research on many topics, including root nutrition, factors affecting lateral root and bud formation, secondary thickening, nodule development, apical dominance, associations with microbial symbionts, biosynthesis of metabolites, selection of resistant and tolerant genotypes and genetic transformation (Bonnett and Torrey, 1965; Torrey and Loomis, 1967; Bonnett, 1972; Budd, 1973; Dodds and Roberts, 1985; Al-Juboory and Skirvin, 1990).

The formation of buds on roots is a well-known phenomenon which occurs in the most successful weeds, and can be used to propagate some plant species by root cuttings (Torrey, 1958; Peterson, 1975; Esau, 1977).

The regeneration of shoots from isolated root cultures has been obtained in various species, the regeneration is generally associated with the addition of exogenous growth regulators and with plants which can produce shoots from roots *in vivo* (Torrey, 1958; Charlton, 1965; Eapen and Gill, 1986; Rybozynski and Badzian, 1987).

In vivo regeneration is a normal fact in sweet potato tuberous roots and can also occur in non-tuberous roots. The tuberous roots are normally used as "seeds" for propagation of sweet potato, where they produce various sprouts which can normally grow from both middle and distal parts, or from any of the parts if the dormancy is broken.

The non-tuberous roots can begin the production of bud primordia as early as four weeks after the growth initiation, the buds arise exogenously in the region of the primary cortex, opposite the xylem rays and sometimes associated with lateral roots (Wilson and Lowe, 1973).

There have been few reports on the formation of buds on *in vitro* sweet potato root cultures. Nakajima and Kawakami (1969) obtained bud primordia formation on 7 out of 110 lateral roots produced from tuberous root cultures treated with extract of tuberous sweet potato roots added to White's minerals with 2% sucrose, no further development was observed. Gunckel and colleagues (1972) obtained a few shoots from callus formed on cultures of 2 mm cylinders from old sweet potato root tubers growing in White's basal medium added by 1 $mg.l^{-1}$ IAA, or 1 $mg.l^{-1}$ GA_3. Better results were obtained by Carswell and

Locy (1984) with excised roots of cultivars Jewel and Caramex produced from calli formed on various organ cultures at 25°C and 16 hour photoperiod on MS basal medium supplemented with BA and NAA (1 and 0.1 $mg.l^{-1}$, respectively); under such conditions many shoots were formed on the roots.

The aim of the following experiments was to induce direct shoot formation on root cultures; the development of such a procedure could be very important, especially for the regeneration of cryopreserved root-tips of sweet potato germplasm (see section 3.2).

4.2 Material and Methods

4.2.1 Plant material

The roots used for tests of shoot induction were excised from the *in vitro* donor plants. The length of roots varied according to their origin and genotype, and there was also a variation among roots of similar origin. Roots of different length and from different parts of the plant (basal and adventitious) were cultured separately in preliminary tests, but as their behaviour did not follow any pattern in line with their origin, the succeeding cultures used all types of roots which were

longer than 1 cm in length. Unless otherwise stated, the roots used for the experiments were obtained from one week old *in vitro* plantlets.

Brondal, Jersey Orange, Papota and TIB-10 were the genotypes used for the root cultures, generally one or two of these genotypes were tested in each experiment.

4.2.2 Methods

The basic medium used for the root cultures was semi- solid (0.6% agar) MII-m (see section 2.2) without kinetin and GA_3 supplemented with NAA, IAA or BA at levels which will be detailed in the following sections. Cultures were grown in 9 cm plastic petri dishes containing 30 ml nutrient medium and sealed with Parafilm M. (American Can Co.). The environmental conditions were $25\pm2^{0}C$ with a 16 hour photoperiod at 30 $\mu Mm^{-2}s^{-1}$ PAR.

4.3 Results

4.3.1 Effects of NAA and IAA on the shoot development of sweet potato root cultures

These experiments tested the effects of NAA at 0.186, 1.86 and 18.6 $mg.l^{-1}$ on root cultures of genotypes Jersey Orange, Brondal and Papota and the effects of IAA at 0.175, 1.75 and 17.5 $mg.l^{-1}$on root cultures of Brondal and Papota.

The results show that neither auxin was effective in increasing the bud formation on sweet potato root cultures.

Only Jersey Orange root cultures treated with a low level (0.186 $mg.l^{-1}$) of NAA showed the formation of two shoots over the total of 12 root samples. The controls of that same genotype, however, showed the formation of three shoots over the same total of samples (see Figure 4.1). The shoots begun to be produced three weeks after the tests started, this period was not affected by the treatments.

All the three genotypes showed very good roots in the controls, their colour was green and they had no callus. The cultures treated with NAA at 18.6 $mg.l^{-1}$ were pale green or cream and brown. There was an increase on the callus production along the roots simultaneously with the increase of auxin concentration.

Generally, roots treated with IAA did not grow, except for a few from Papota at 0.175 $mg.l^{-1}$ and Brondal at 1.75 and 17.5 $mg.l^{-1}$, otherwise, almost all of the original roots developed short lateral roots. NAA treated roots showed some growth in length and produced lateral roots at the lower concentrations only (0.186 and 1.86 $mg.l^{-1}$); this effect decreased with the increasing concentrations of the auxin and at 18.6 $mg.l^{-1}$ there was no growth and no lateral root development, especially with Papota and Brondal, although Jersey Orange showed many very short lateral roots but no growth inmain axis length.

High levels of either NAA or IAA induced some thickening on the lateral roots. Genotype Brondal showed thick lateral roots at all levels of either NAA or IAA. Genotype Papota treated with NAA or IAA showed short and curly lateral roots at 1.86 and 18.6 $mg.l^{-1}$. Higher levels of NAA or IAA were damaging to the development of the roots, stopping or reducing their growth, reducing the production and the quality of secondary roots and affecting the geotropism.

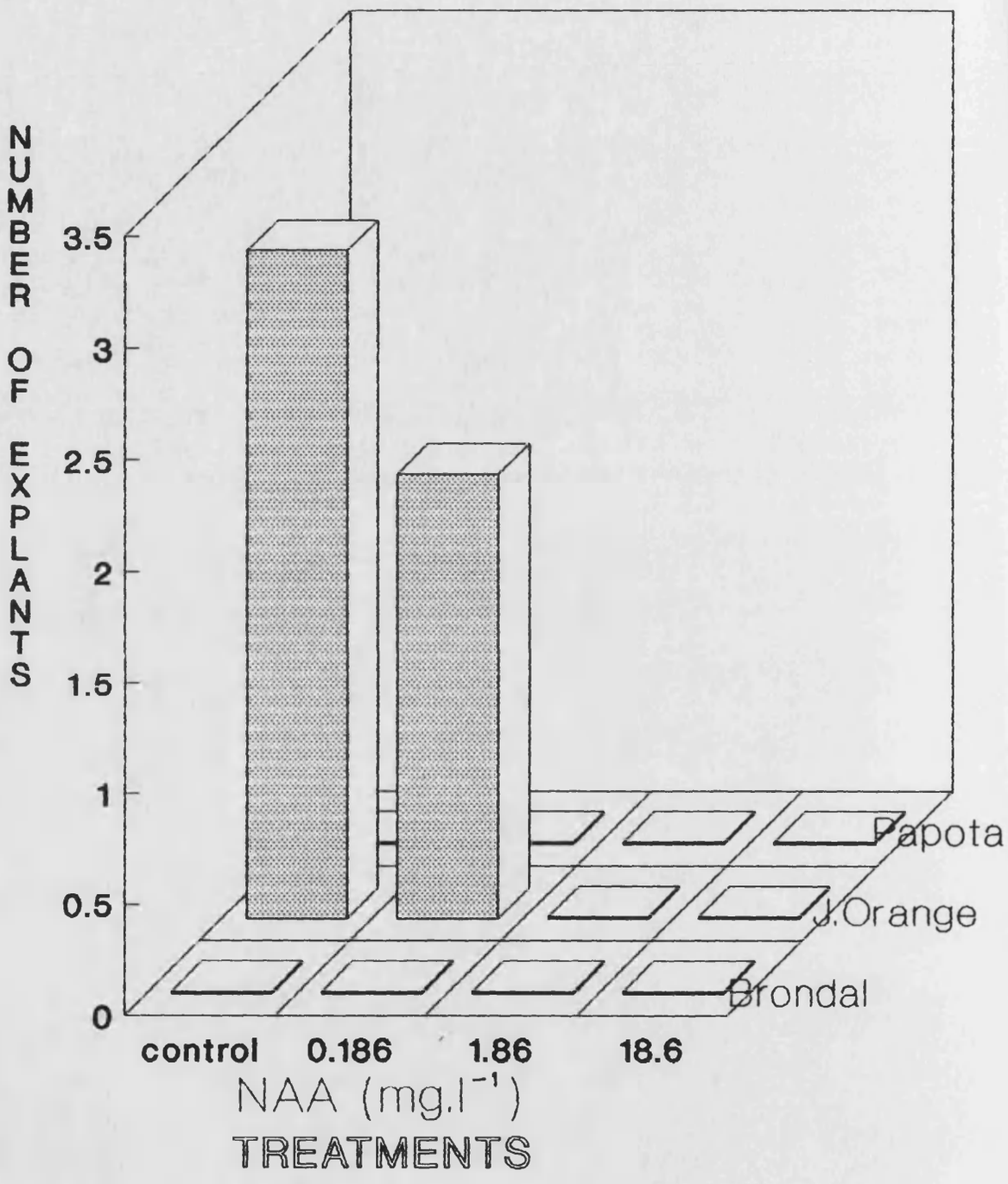

NUMBER OF EXPLANTS
3.5
3
2.5
2
1.5
1
0.5
0
Papota
J.Orange
Brondal
control
0.186
1.86
18.6
NAA (mg.l⁻¹)
TREATMENTS

Figure 4.1 Effects of NAA on the induction of adventitious shoots on root cultures of Brondal, Jersey Orange and Papota. The data were collected from six week cultures and show the total number of explants which produced adventitious shoots.

Key:

Number of replicates: 12

Explants: roots from 1 week old plants

Basic medium: MII-m without GA_3 and kinetin

Procedure: 6 weeks on NAA 0.186, 1.86 and 18.6 $mg.l^{-1}$

Temperature: 25±2°C

Light conditions: 16 hour photoperiod

30 $fMm^{-2}s^{-1}$PAR

4.3.2 Effects of NAA and BA on the development of shoots on three types of sweet potato root culture.

This experiment was based on the results obtained by Carswell and Locy (1984) on Jewel and Caramex sweet potato genotypes. Their results showed shoot production from root cultures which were regenerated from leaf, stem and root tubers treated with NAA and BA at 0.1 to 10.0 $mg.l^{-1}$. Their best results were obtained at 1 $mg.l^{-1}$ NAA and 0.1 $mg.l^{-1}$ BA.

The levels of NAA and BA tested here were 0, 1, 2 $mg.l^{-1}$ and 0, 0.1, 0.2 $mg.l^{-1}$, respectively. The genotype was TIB-10, which had produced a few shoots in a preliminary experiment using MII-m . The roots were taken from three-week old stock shoot cultures. Three types of explants were tested: **a**) whole 10 cm roots, **b**) 10 cm roots cut into 1 cm segments and **c**) 1-2 cm aerial adventitious roots. The secondary roots which were already developing on the explants **a**) and **b**) were not cut off from the main root in order to avoid callus formation on the wounds. The 1 cm root segments were laid on the petri dishes keeping the natural sequence in a way that their position could be easily identified along the main root.

The results collected at four and eight weeks after the experiment began showed the formation of shoots from all the types of explants. The use of BA alone or interacting with NAA did not induce the shoot formation under the tested conditions. The shoots were only formed

variable according to the type of root and treatment. The development of shoots was independent of the concentration of NAA, however the number of shoots decreased with the increase of NAA level (Figure 4.2). The number of shoots obtained by cultures treated with 1.0 $mg.l^{-1}$ NAA and the controls were similar, but the control was better. Most of the shoots were produced from a lateral root branch point (Plate 4.1-c). The shoots were formed on the main root and also on the lateral roots.

Almost all the shoots developed normally when transferred to fresh MII-t, with the larger shoots showing the most rapid development (Plate 4.3-a,b), and the very small shoots require a long time to develop in plantlets.

The shoot formation seemed to be independent of the length of the main root or the number of secondary roots produced or their length (Plate 4.1-a,b), but the short adventitious roots seem to have less possibility of producing shoots.

All the cultures treated with growth regulators produced callus. The callus production was directly proportional to the increasing concentration of growth regulators (Plate 4.2). The callus were hard and some of them very compact, forming structures similar to galls, but none of the shoots arose from the callus. Some of the roots showed a thin layer of callus along them.

There were differences between the responses of the three types of explants to the treatments. The quickest

three types of explants to the treatments. The quickest results were obtained with the whole roots which started producing shoots two weeks after culture initiation and a total of six shoots (both control and 1 $mg.l^{-1}$ NAA treatments produced 3 shoots each) from the 36 explants of the three treatments which showed shoots at the end of eight weeks. These shoots showed different stages of development, varying from 0.2 cm buds to a 10 cm fully developed plantlet. Some roots produced more than one shoot and shoots were formed on both main and secondary roots.

The 1 cm root segments produced 10 shoots (5, 4 and 1 from control, NAA 1 $mg.l^{-1}$ and NAA 2 $mg.l^{-1}$, respectively) over the total of 36 explants of the three

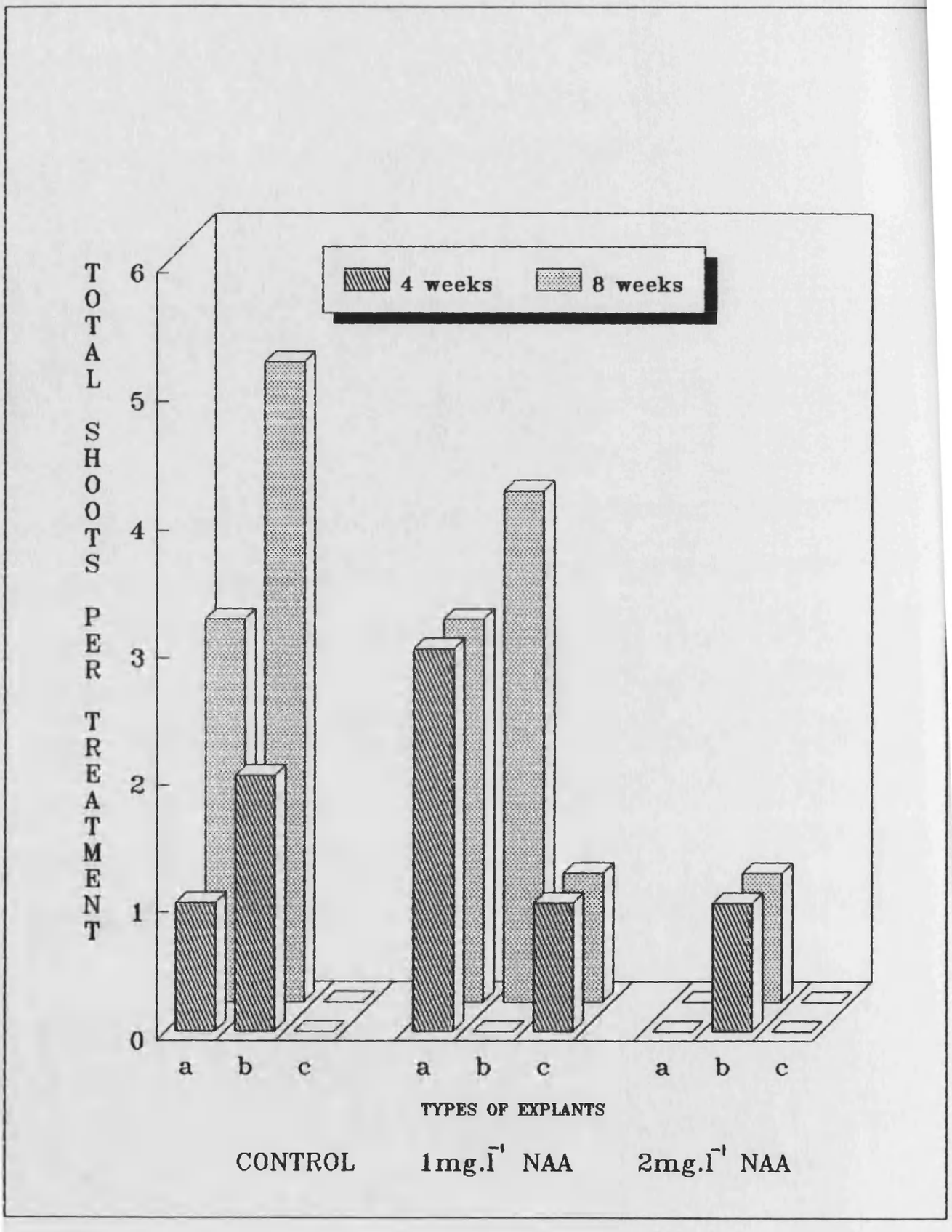

4 weeks
8 weeks
TOTAL SHOOTS PER TREATMENT
0
1
2
3
4
5
6
a b c a b c a b c
TYPES OF EXPLANTS
CONTROL
1mg.l⁻¹ NAA
2mg.l⁻¹ NAA

Figure 4.2 Effects of NAA and BA on the development of shoots on TIB-10 root cultures

Key:

Number of replicates: 12

Explants: **a**) 10 cm roots, **b**) 1 cm segments from 10 cm roots, **c**) 1 cm adventitious roots

Basal medium: MII-m without growth regulators

Procedure: 8 weeks on 0, 0.1 and 0.2 $mg.l^{-1}$ BA

0, 1 and 2 $mg.l^{-1}$ NAA

Temperature: $25\pm2^{0}C$

Light conditions: 16 hour photoperiod

30 $\mu Mm^{-2}s^{-1}$PAR

Plate 4.1

a

b

c

Plate 4.1 **Adventitious shoots growing from genotype TIB-10 root cultures treated with 0.1 mg.l^{-1} NAA at 25±2^{0}C.**

a) and **b)** Cultures under the same treatment showing uneven root development, but both with a well-developed shoot. The shoot formation did not depend on the quantity of roots.

c) Detail of a shoot growing from a lateral root branch point (x 1.35)

Plate 4.2

a

b

c

Plate 4.2 Effects of concentration of NAA on the development of callus on genotype TIB-10 root segment cultures. Despite the callusing, the shoots were formed from the roots.

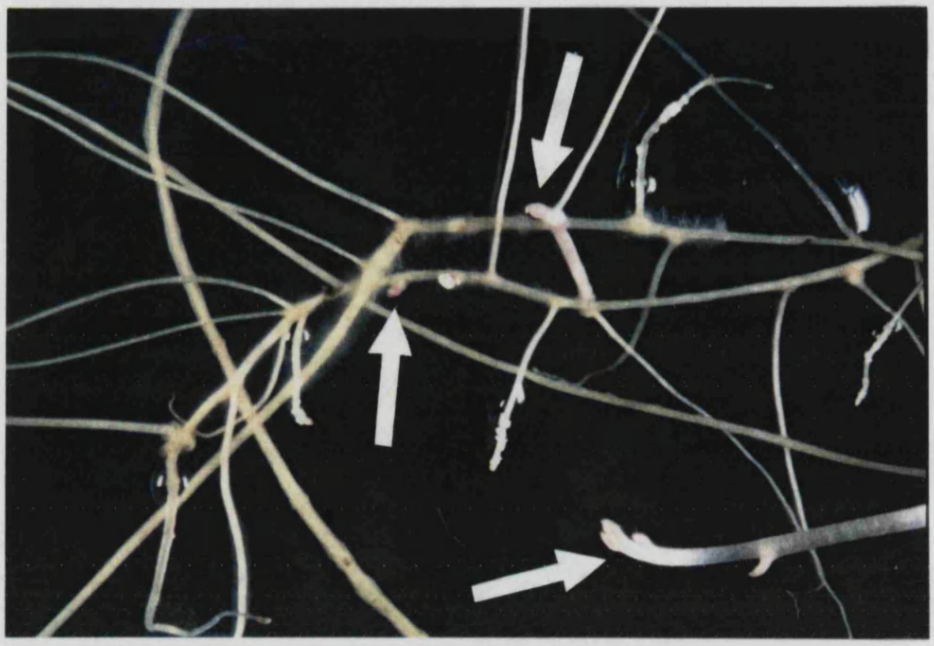
a) Control (x 2.2).

b) 0.1 mgl^{-1} NAA (x 0.85).

c) 0.2 mgl^{-1}NAA (x 0.85).

Plate 4.3

a



b

Plate 4.3 Effects of proximal distance on the shoot development and regeneration. Note that the more proximal the shoot, the better and faster are the development and subsequent regeneration.

a) A 21 day old genotype TIB-10 root culture developing three shoots distributed along the same primary root. Note the difference of sizes among these shoots. (x 0.85)

b) The same shoots 7 days after being sub-cultured.

4.4 Discussion

Although isolated roots of many species have been routinely cultured by many researchers since the development of such technique by White in 1934, most of them used techniques where shoots are obtained via callus (see review by Peterson, 1975). There were, however, a few species reported to produce buds or shoots not related to callus formation: *Convolvulus* sp (Torrey, 1958; Bonnett and Torrey, 1965), *Linaria vulgaris* (Charlton, 1965), *Ipomoea batatas* (Carswell and Locy, 1984), *Vigna radiata* (Mathews and Rao, 1984), *V. aconitifolia* (Eapen and Gill, 1986), *Lotus corniculatus* (Rybozynski and Badzian, 1987).

The results of the experiments presented in this chapter confirmed the results obtained by Carswell and Locy (1984) that it is possible to regenerate shoots directly from root cultures of sweet potato. Shoots were produced under various conditions, although it is not yet clear which factors stimulate this phenomenon.

The root cultures of TIB-10 and Jersey Orange developed shoots when treated with a low level (1 $mg.l^{-1}$ and 0.186 $mg.l^{-1}$, respectively) of NAA; Peterson (1975), pointed auxins as inhibitory to shoot formation but with some exceptions, low auxin levels can be favourable to bud initiation; however it is not clear that the use of NAA was an important factor on the shoot formation of sweet potato root cultures because there was shoot formation on the controls.

The apparent inhibition of shoot formation caused by BA at the levels tested, the decrease of the number of shoots when NAA was increased and the shoot proliferation in the controls are factors which can indicate that the shoot production was stimulated by other factors than by exogenous growth regulators. Few other species are reported to produce shoots on root cultures in hormone-free medium, generally this phenomenon is related to species which can present vegetative propagation from roots in natural conditions (Charlton, 1965; Eapen and Gill, 1986; Rybozinsky and Badzian, 1987).

CHAPTER 5

CHARACTERIZATION OF GENOTYPES USING ISOZYME ELECTROPHORESIS

5.1 Introduction

Isozymes are multiple molecular forms of an enzyme located in a single organism; such forms have either similar or identical catalytic activities, and are coded by more than one gene locus. The term isozyme can also be taken to include allozymes (enzymes coded by different alleles at a single locus) (Simpson and Withers, 1986).

Isozymes are present in all animal and plant organisms and they were first discovered in the late 1940's. The techniques for their investigations were improved during the next decades with the development of filter paper and then starch gel electrophoresis and the demonstration, that it was possible to visualize enzymes on those supporting media when a specific histological stain was applied (see review by McMillin, 1983). Later, techniques for other types of supporting media were developed including cellulose acetate, agarose and polyacrylamide gels (Brewer, 1970).

Nowadays gel electrophoresis is a widely used chromatographic technique for separations of mixtures of ionic compounds in preparative and analytical biochemistry and many areas of research. Gel electrophoresis combines elements of free-boundary

electrophoresis (separation based on electrical charges) and gel filtration (separation based on molecular sizes).

The enzyme activity staining is based on any reaction or set of reactions that will reveal a zone of enzyme activity by any chemical and/or physical means (Vallejos, 1983). The array of bands developed in the gel is called a zymogram.

Isozyme electrophoresis is an ideal tool for the evaluation of plant germplasm collections as it provides a description relatively unaffected by the environment and is economical and simple to use. However this technique cannot be used as a substitute for the morphological and agronomical evaluations, as these evaluations are of extreme importance for the breeding programmes. The identification and characterization of large collections of germplasm using only morphological and agronomical evaluations is, however, a very expensive, difficult and time consuming process. The costs of cultivation of the huge areas of land required, the quantity of morphological data and the patterns of variation among the genotypes and also the possible variations caused by the agro-climatic conditions make for a very complex programme (Simpson and Withers, 1986; Ramirez *et al.*, 1987).

As isozyme electrophoresis can give more direct genetic information, such as the genetic markers, it may be used for a preliminary characterization of the genotypes facilitating the removal of duplicates,

evaluating the level of genetic diversity and grouping similar accessions for a subsequent field evaluation.

The purpose of the following experiments was to develop an isozyme electrophoretic technique which could be helpful in the characterization and identification of sweet potato germplasm.

5.2 Material and Methods

5.2.1 Plant material

Electrophoretic experiments were separated into two phases: **a**) definition of methodology, **b**) characterization of seven genotypes using the methodology thus defined.

During the first phase various tissues were tested as follows: young leaves, shoot-tips and fibrous roots from *in vivo* plants; whole plants from *in vitro* plants and fibrous roots from *in vitro* root cultures (see section 2.4). Various staining methods were tested and the details of those which were successful and therefore employed in the experimental work are given in Appendix 5 (see also section 5.3.1 and tables 5.1, 5.2, 5.3). At least two genotypes were tested on each gel in order to observe the variability of band patterns among them.

The second phase analysed the seven genotypes (see section 2.1) for their characterization, using the esterase enzyme system alone because of difficulties encountered with the other enzyme systems. The enzyme

system analysed was the esterase; the tissues used were roots grown *in vitro*.

The adventitious roots (0.3-0.4/0.1-0.15 cm length/diameter) which were still covered by the stem epidermis were cultured every two days over an eight-day period, the explants were. Four 100 ml

jars containing 30 ml liquid MII-m without growth regulators were set for each of the seven genotypes; the cultures were grown under normal conditions (see section 2.4.1.3). Samples were taken from each one of the genotypes during four weeks at regular intervals of a week. Following this routine each one of the gels screened roots at different stages of development, from 7 up to 37 days old.

5.2.2 Preparation of samples

The fresh tissues were washed with distilled water and ground with a chilled buffer in a proportion of 1:2 (w:v). The weight of tissues varied according to their origin, as follows: 40 g of material from *in vivo* cultures and about 2 g of the material from *in vitro* cultures (as *in vitro* material are extremely susceptible to dehydration, it is very difficult to take the exact measure), in the case of root cultures, four of them were taken from each culture and homogenised respecting the proportion of 1:2 (w:v).

The homogenization was made using a mortar and pestle maintained inside a polystyrene box containing crushed ice, in order to keep the temperature low, thus minimizing unwanted denaturation of proteins. Three non-denaturate extraction buffers were tested: **a**) Buffer-I: 50 mM tris-acetate pH 7.5, 1 mM EDTA, 1% ascorbic acid, 0.5 M sucrose (Maeshima *et al.*, 1985; **b**) Buffer-II: 50 mM tris-HCl pH 8.3, 5% PVP-40, 0.5% Triton X-10, 14 mM

mercaptoethanol, 20% sucrose (Ramirez *et.al,* 1987); **c)** Buffer-III: 50 mM Na_2PO_4 pH 7.0, 6 mM dithiothreitol (Shields *et. al*, 1983). Buffer I was used originally by Maeshima and colleagues (1985) for extracting sweet potato proteins from tuberous roots, but buffer II was originally used for cassava by Ramirez and colleagues (1987) and buffer III was used for *Eucalyptus* sp by Shields and colleagues (1983).

The homogenate was centrifuged at 13.000xg for 10 minutes at 5^0C. The supernatant was removed and placed into a separate container; to this was added, a 60% (v/v) solution of glycerol containing 0.002% (w/v) bromophenol blue equivalent to one fifth of the volume of supernatant, at this point the samples were ready to be loaded onto the gel.

5.2.3 Preparation of polyacrylamide gels

A high pH non-dissociating discontinuous buffer system was used for the polyacrylamide (acrylamide:bisacrylamide at 30:0.8) gels as described by Davis and Orstein (1964) (Appendix 4). The system requires two gel layers of different pH and polyacrylamide concentration.

The gel mixtures (Appendix 3) were prepared immediately before pouring them into the 16 x 16 cm glass plates, following the procedures of Hames and Rickwood (1981). The 20 ml resolving gel mixture (Tris-HCl pH 8.8 (see Appendix 4), with a variable concentration of

polyacrylamide) was poured first into the plates and left to polymerize for 45-60 minutes. The concentrations of polyacrylamide on the resolving gel were 7.5%, 10% and 12.5%. The 5 ml stacking gel mixture (Tris-HCl pH 6.8 (see Appendix 4), with 2.5% polyacrylamide) was poured over the polymerized resolving gel. A comb was inserted on the top of the stacking gel in order to form the wells where the samples were loaded later.

The numbers of wells used were 15 or 20, according to the number of samples analysed. Two hours later, the wells were washed with distilled water and the chilled samples were carefully loaded by a micro syringe fitted with a long blunt needle. The volumes of samples were 20 or 40 ul, for the 15 or 20 wells, respectively.

5.2.4 Electrophoresis procedure

The electrophoresis was carried out in a cooled Protean-II vertical slab apparatus connected to an electrophoresis power supply ATTA-AE-3105 with 500V-500 mA capacity. The power pack was adjusted at 30 mA and variable voltage; at this calibration the boundary could migrate 13 cm in 3 hours. The reservoir buffer was Tris-glycine pH 8.3 (Appendix 4).

5.2.5 Isozyme staining and evaluation

Once the electrophoresis was completed there was no need for keeping the temperature low, but the staining

procedure had to be carried out immediately in order to avoid a diffusion of the bands.

In the first phase of experiments, various enzyme staining techniques were investigated, but only a few of them were deemed to be successful (see Tables 5.1, 5.2 and 5.3 and Appendix 5).

Following these results, the second phase was based on esterase isozymes of the *in vitro* roots extracted using Buffer-III and stained with the method developed by Kahler and Allard (1970) as described in section 2.4.2

The zymograms were analysed visually. This analysis looked at the relative electrophoretic mobility (Rfs) of bands, band quality, stability, and repeatability of results.

5.3 Results

5.3.1 Development of techniques

As there were no specific techniques available for the isozymic characterization of sweet potato, various methods which were successful in other species, were tried on the sweet potato genotypes with some positive results as can be seen below in the Tables 5.1, 5.2 and 5.3. These techniques were tested on tissues of nodal segments, shoot-tips, young leaves and roots collected from *in vivo* stock of plants and roots and whole plants originated from *in vitro* stock of plants.

At first the gels were made with 7.5%, 10% or 12.5% polyacrylamide, however at lower concentration they did not produce sharp bands and, moreover, they were difficult to manipulate because of their fragility. Consequently, the gels were used at 10% and 12.5% concentration; eventually, as the results were quite similar for both concentrations, the gels were made with 10% acrylamide.

All the three extraction buffers tested produced at least a few good zymograms for specific tissues and enzyme systems (tables 5.1, 5.2 and 5.3). However, Buffer-III which was originally developed for *Eucalyptus* tissues (Shields *et. al.*, 1983), proved to be efficient at producing good extracts from all the sweet potato tissues tested in these experiments, generating good zymograms for most of the enzyme systems tested.

Various staining methods were tested on specific enzyme systems using three different extraction buffers; only a few of them gave good or reasonable banding. The more effective methods were selected (Appendix 5) and used for different tissues in order to evaluate the quality and stability of the bands produced.

Esterase (EST), glutamate-oxaloacetic transaminase (GOT), leucine amino peptidase (LAP), peroxidase (PER) and acid phosphatase (AcPh) were the enzyme systems which showed the better results, although these results varied depending on the tissue analysed and the extraction buffer that was used (Tables 5.1, 5.2, 5.3). Although PER and EST produced good or very good zymograms for all the

tissues tested, PER bands faded with time, while EST electrophoregrams could be easily preserved in dried gels, without losing the quality of zymogram patterns.

Young leaves generally showed satisfactory quality of bands for most of the isozymes tested, especially EST, although they did not show stability when analyses of EST were repeated with different leaf samples.

Shoot-tips also showed unstable patterns in a similar way to the young leaves.

There were differences in the patterns produced from *in vivo* and *in vitro* roots and also there were differences in the quality of bands produced when both tissues were tested with the same methods. Tissues originated from *in vitro* cultures produced much better zymograms than any others; their stability was obvious and consequently the gels could be repeated. The use of such roots was therefore the most practical approach because these tissues are easily produced, their use does not damage the donor plant and contaminations can be controlled in a better way than when using *in vivo* plants.

In vivo tissues	% acryl.	Isozymes EST	GOT	LAP	PER
Shoot-	7.5	*	--	--	*
tips	10.0	*	*	*	*
	12.5	+	*	*	*
Young	7.5	*	*	*	*
leaves	10.0	+	+	-	++
	12.5	*	*	*	*
Nodes	7.5	+	+	*	+
	10.0	*	*	*	*
	12.5	++	+	*	*
Roots	7.5	*	*	*	*
	10.0	+	+	-	++
	12.5	*	*	*	*

Table 5.1 Effects of Buffer-I (Ramirez et al., 1987) on the banding of various tissue extracts stained for four enzyme systems. Note: these are the results of various gels where various genotypes were tested

Legend

-- no bands or stain

- stained lanes and/or very weak bands

+ good bands

++ very good bands

* missing values

In vivo tissues	% acryl.	Isozymes EST	GOT	LAP	PER
Shoot-tips	10.0	+	+	*	++
	12.5	++	*	*	*
Young leaves	10.0	++	+	-	++
	12.5	*	*	*	*
Nodes	10.0	*	*	-	*
	12.5	*	*	*	*
Roots	10.0	++	+	-	++
	12.5	*	*	*	*
In vitro roots	10.0	+	*	*	++

Table 5.2 Effects of Buffer-II (Maeshima et al., 1985) on the banding of various tissue extracts stained for four enzyme systems. Note: these are the results of various gels where various genotypes were tested

Legend

-- no bands or stain

- stained lanes and/or very weak bands

+ good bands

++ very good bands

* missing values

In vivo tissues	% acryl.	Isozymes EST	GOT	LAP	PER	AcPh
Shoot-tips	10.0	++	+	++	*	-
Young leaves	10.0	++	+	+	*	+
Nodes	10.0	++	+	*	*	++
Roots	10.0	++	*	+	+	+
In vitro roots	10.0	++	+	++	+	++
In vitro plants	10.0	++	*	*	++	*

Table 5.3 Effects of Buffer-III (Shields et. al., 1983) on the banding of various tissue extracts stained for five enzyme systems. Note: these are the results of various gels where various genotypes were tested

Legend

-- no bands or stain

- stained lanes and/or very weak bands

+ good bands

++ very good bands

* missing values

5.3.2 Characterization of genotypes

The principal aim of the experiments on electrophoresis was the development of a technique for the characterization of the seven sweet potato genotypes. The expectation was that this technique might be useful in the manipulation of banks of germplasm helping in the identification of duplicates and the grouping of similar genotypes prior to field evaluation.

The characterization of the genotypes was based on the results obtained on the previous phase. The tissues used were roots grown *in vitro*, the extraction was made with Buffer-III (see section 5.2.2) and the staining after the electrophoresis was for EST, using the method developed by Kahler and Allard (1970) (Appendix 5).

The root cultures were initiated every two days over an eight day period, as it was described in section 5.2.1. Samples of four roots were taken from each one of the genotypes and prepared with extraction Buffer-III at the proportion of 1:2 (w:v). The gels were repeated within a regular interval of a week from each other during four weeks in a manner such that the roots were analysed at different stages of development, from 7 up to 37 days old.

The results (Table 5.4 and Plates 5.1 and 5.2) showed only one EST zone which is of fast migration. The seven tested genotypes are quite heterogeneous showing a good genotype specifity which could be repeated. The band

with Rf=0.66 is common to all the genotypes, another band (Rf=70) is common to six genotypes except TIB-10. Other bands are common to more than one genotype but each one of them showed a unique array of bands.

The zymograms showed a good stability in the patterns with no differences among them during a month of culture. But after this month there was a change in the Rfs of the faster bands of Brondal, TIB-10 and Rose Centennial (see Appendix 6, 10 and 11, respectively) which became slightly slower.

Table 5.4 Zymogram of the esterase isozymes of sweet potato roots cultivated *in vitro*

Key:

Plant material: 7 to 30 day-old roots

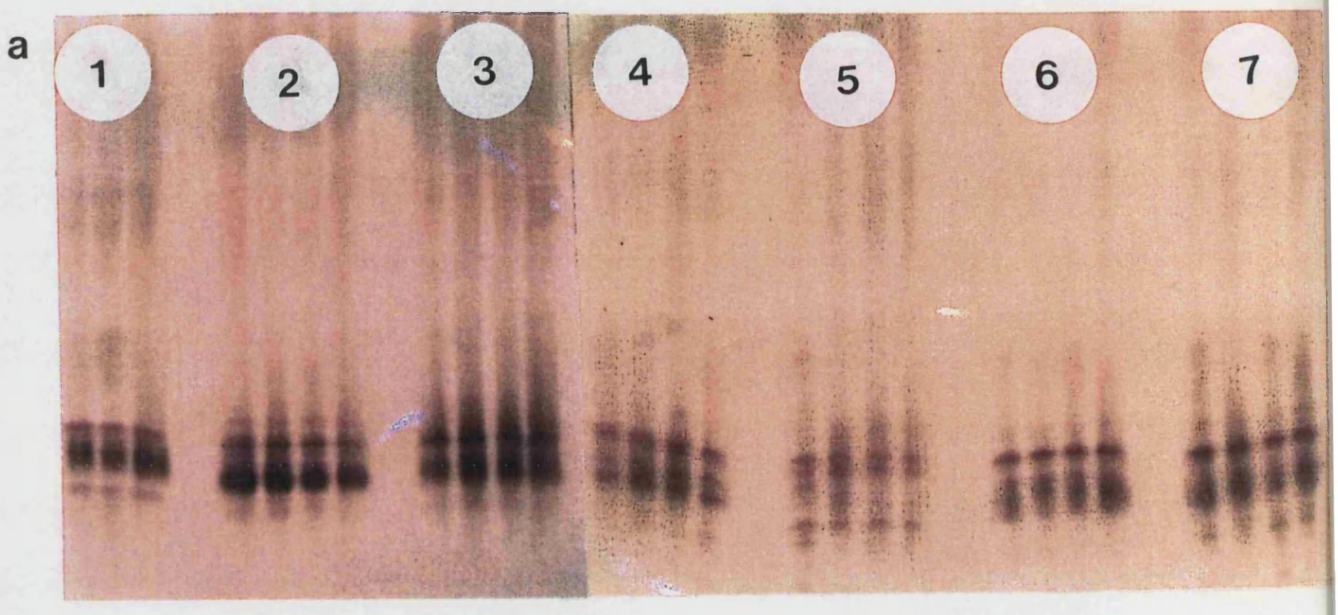
Procedure:

a)Samples:4 roots were homogenised per genotype with Buffer-III (Appendix 4), centrifuged and then samples of 20 ul were analysed in each gel

b)Staining: Esterase as described by Kahler and Allard (1970)(Appendix 5)

Note: Rf= distance migrated by protein divided by distance migrated by the dye (Hames and Rickwood, 1981)

Plate 5.1



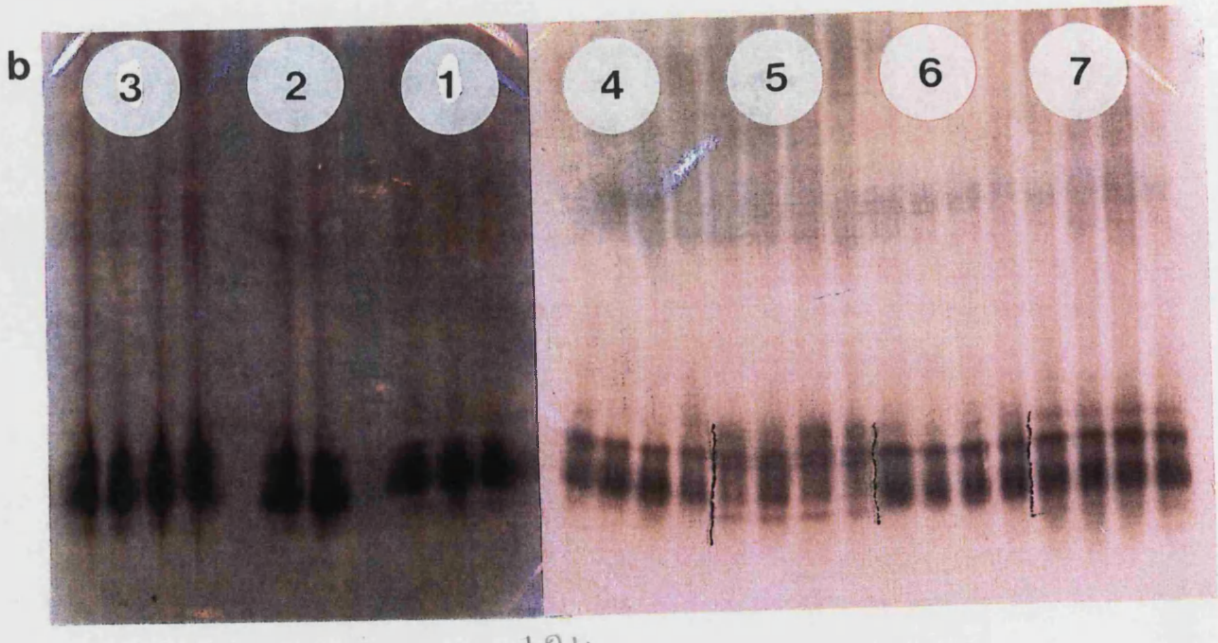
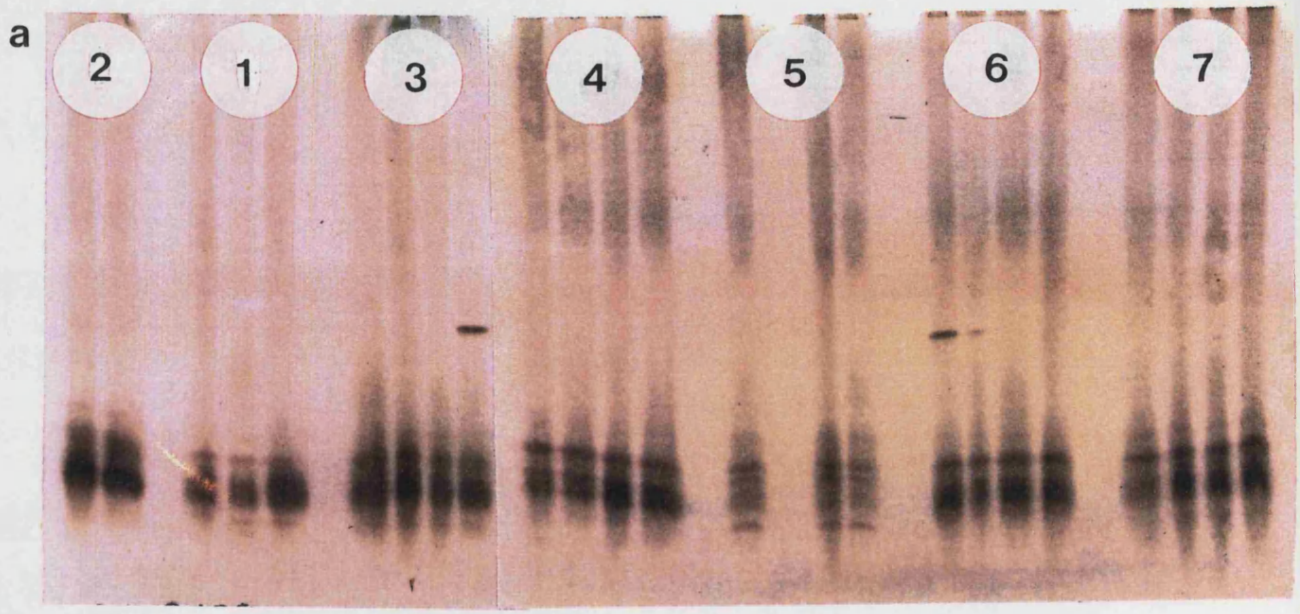


Plate 5.1 Esterase zymograms of sweet potato *in vitro* roots following the sampling of cultures at two-day intervals between 7 to 21 days. Seven genotypes are characterized: 1) Brondal, 2) CN-1367-2, 3) Jersey Orange, 4) TIB-9, 5) TIB-10, 6) Papota and 7) Rose Centennial. (see also Plate 5.2). Note: there were some missing samples because contamination of the cultures by bacteria.

a) Seven to thirteen day old root cultures.

b) Fifteen to twenty one-day old root cultures.

Plate 5.2



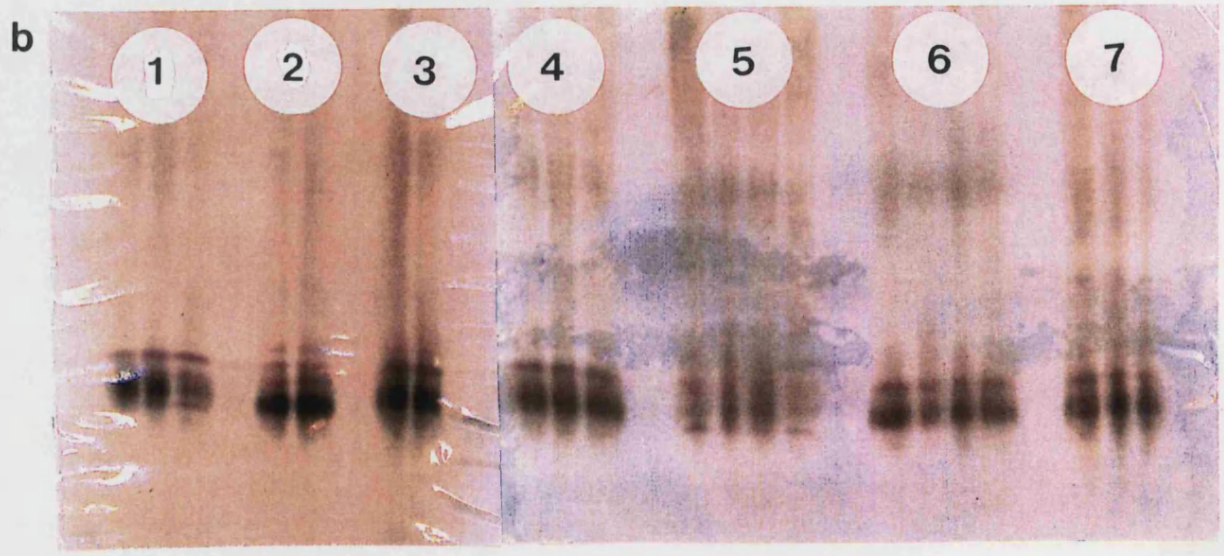


Plate 5.2 Esterases zymograms of sweet potato in vitro roots following the sampling of cultures at two-day intervals between 23 to 37 days. Seven genotypes are characterized: 1) Brondal, 2) CN-1367-2, 3) Jersey Orange, 4) TIB-9, 5) TIB-10, 6) Papota and 7) Rose Centennial. (see also Plate 5.1). Note: there were some missing samples because bacteria contamination of the cultures.

a) Twenty-three to twenty-nine day old root cultures. Note: the slow bands on genotypes 3 and 6 are the result of a bacterial contamination.

b) Thirty-one to thirty-seven day old root cultures.

5.4 Discussion

The electrophoresis of isozymes can be a useful tool in the characterization of germplasm. One of the major advantages of using this technique is the speed with which the analyses can be carried out on a large number of plants, using small sample volumes (Simpson and Withers, 1986; Ramirez *et. al.*, 1987). The technique is especially important in the preliminary evaluation of collections, helping in the identification of replicates and also in the identification and selection of groups of genotypes.

The aim of these experiments was to develop a technique which could be used for a fast identification of sweet potato germplasm.

Ortega (1987) developed techniques for the characterization of sweet potato genotypes which were based on the electrophoretic patterns of proteins and isozymes of the tuberous roots; also Huaman and De la Puente (1988), mentioned the research which had been conducted by Stegemann at the German Agency for Technical Cooperation (GTZ) in Germany using proteins and esterases of tuberous roots for the verification of duplicates. There is no doubt that those methods are effective and can be very good alternatives when root cultures are not possible, however, tuberous roots are necessary in order to perform the technique. The choice of *in vitro* root cultures or *in vivo* tuberous roots depend on the availability of one or other plant material.

Esterase isozymes from sweet potato roots grown *in vitro* proved to be a good solution for the fast identification of genotypes since the zymograms showed specific array of bands for each of the genotypes which could be repeated from 7 to 30 day-cultures.

There are many advantages in using root cultures for the characterization: **a**) roots are easily available during almost all of the life cycle of the sweet potato plants, **b**) root cultures are easily produced in a short period of time, not requiring complicated techniques, **c**) as only roots are used, the plants can be maintained without damaging their viability as characterization goes on, **d**) as the zymograms showed stability among cultures from a week to a month old, there is no hurry to use the donor cultures, the electrophoresis analysis can be adequately programmed within a month, using the same cultures, **e**) as plants do not need to be cultured in the soil, the method is relatively cheap, **f**) as the cultures are kept in small containers in the laboratory, there is economy of space during the culture and analysis period, **g**) the genotypes are protected against environmental and pest/disease conditions.

Despite of all of these advantages, isozymes are limited in the level of polymorphism and number of loci that can be detected; it is safer to use electrophoresis in a manner that it is complementary to standard methods of characterization involving quantitative and qualitative descriptors (Withers and Simpson, 1986; Ramirez *et al*, 1987; Bernatsky and Tanksley, 1989).

CHAPTER 6

GENERAL DISCUSSION

Sweet potato is one of the world's most important food crops (FAO, 1990); its potential as a source of raw material for the industrial production of starch and alcohol, foods and pharmaceutical industry is incompletely exploited (Villareal *et al.*, 1979; Collins and Walter, 1982; Horton, 1988; Horton *et al.*, 1989; Florkowski and Jarret, 1990; Jarret and Florkowski, 1990).

The variability amongst *I.batatas* is extensive and breeders have only begun to exploit it. It is well known that the availability of collections with a wide genetic diversity is one of the most important steps for the improvement of the genotypes (Simpson and Orgonzaly, 1986; Cohen, 1989; Withers and Williams, 1990), and this is also the most important factor which makes sweet potato germplasm conservation a necessity as it has been pointed out by many scientists (Huaman and De la Puente, 1988; Horton *et. al.*, 1989; Florkowski and Jarret, 1990; Jarret and Florkowski, 1990).

Storage is only one aspect of germplasm conservation of vegetatively propagated crops where *in vitro* techniques can be applied; the IBPGR (1986) has related such techniques to activities associated with collection, disease indexation, quarantine, multiplication,

characterization and evaluation, storage and distribution of germplasm.

This thesis is a contribution to the existing studies on the conservation of sweet potato germplasm. It involves three aspects of the *in vitro* germplasm conservation activities proposed by the IBPGR Advisory Committee on *in vitro* storage (IBPGR, 1986), as follows: **a**) characterization using esterase electrophoretic patterns of root cultures, **b**) multiplication of root and shoot cultures and **c**) storage of shoot segments under oil-overlay and cryopreservation of root-tips. The present section discusses the results obtained from those studies, focusing on their advantages and their problems and also indicating, where possible, the use of those results as models for other species which present similar problems of conservation.

The use of oil-overlay is widespread as a method which can decrease the activity of microorganisms to almost zero for long periods of time (Buel and Weston, 1947; Caplin, 1959). Not only is it very cheap and effective, but it is also an extremely simple method which does not require special apparatus, physical conditions or specialised personnel. Despite all these advantages, only a few researchers have tested the method on the storage of plant tissues: Caplin (1959) with calli of carrot and grape; Augereau and colleagues (1986) with calli of *Catharanthus roseus*, *Amsonia tabernae-montana*, *Atropa beladona*, *Papaver sonniferum*, *Rosmarinus officinalis*, *Vinca minor*, *Voacanga thoursii*,

Coffea arabica and *Glycine max*; Moriguchi and colleagues (1988) with calli of grape; Mannonen and colleagues (1990) with cell cultures of *Panax ginseng* and *Catharanthus roseus*; and Mathur and colleagues (1991) with buds of *Valeriana wallichii*, leaves of *Nicotiana tabacum*, calli of *Hyoscyamus muticus*, *Parkinsonia aculeata* and *Plantago major* and calli and cell cultures of *Selenium candolii*. To date the storage under oil-overlay has not been reported as a method for the conservation of IVAGs of vegetatively propagated crops.

The results described in section 3.1, show intervals of 12 and 17 months between sub-cultures for cultivars Papota and TIB-10 and CN-1367-2, respectively, maintained under LP and LS. Allowing for the expected variation in responses of different genotypes, these results are similar to the 13 months reported by Allan (1979), the 8 and 12 months obtained by Desanero and Rhodes (1989) and the 12 months by Jarret and Gawel (1991), with different genotypes of the same species stored under different conditions. Although the storage under oil-overlay did not produce dramatically better results than the other procedures, it is especially advantageous because of its low cost, simplicity and security, characteristics which are a priority for both developed and less-developed countries (Florkowski and Jarret, 1990; Jarret and Florkowski, 1990; Huaman and De la Puente, 1988).

Withers (1987) criticised the method as lacking in practicability and convenience, despite considering it

essentially uncomplicated in concept. Experience has shown that the practicability depends on the development of a well organized procedure; for instance, the oil addition seems at first to be a cumbersome procedure, nevertheless the use of a disposable syringe can make it a reasonably clean operation. The possibility of performing both the subculturing and the oil addition at the same time is another practice which has proved to be convenient. Another possibility for simplifying the method was proposed by Caplin (1959) who suggested that the medium and the oil could be sterilized simultaneously. This possibility was not tested with the nodal segments, although it would appear to be feasible, particularly in laboratories where the distribution of medium into the test tubes is made before the sterilization.

Nevertheless, as these were the first trials, the research must be continued in order to test a greater number of genotypes and species, to check interactions of the oil-overlay with different physical and nutritive conditions and especially to look at techniques to improve the method for recovering the plants after storage. Studies on the genetic stability would also be important.

Although the use of cryobiological methods for the storage of germplasm would undoubtedly be the most secure way to maintain the genetic stability of genotypes on a long term basis (Henshaw, 1975; IBPGR, 1983; Scowcroft, 1984; Withers, 1987), such freezing procedures with

organized tissues present technical and biological problems which have been delaying the development of routines for this type of conservation. Successful cryopreservation of meristems of species such as potato, strawberry and carnation has been reported by several workers (see reviews by Kartha, 1981; Kartha, 1985; Bajaj, 1987; Withers and Williams, 1990), but, rather than pointing the way to success, these reports simply prove that generalizations are not readily made and no single routine can be recommended because each species reacts differently to this method (Withers and Williams, 1990).

It has been reported that sweet potato meristems are very difficult to cryopreserve (Kadir and Rhodes, 1989), and the results of experiments in section 3.2 of this thesis endorsed this view, since shoot meristems did not survive at temperatures below -10°C. Recently, however, Towill and Jarret (1992) obtained up to 64% survival of shoots after cryopreservation using the technique of vitrification. Vitrification is a procedure in which very high cooling rates are employed to achieved the transformation of water to a vitrified or glass state, rather than to the more normal crystalline state. The temperature at which this transformation occurs may be lower than -100°C, but it is higher with increased solute concentrations. One problem, therefore, is the avoidance of toxicity when sufficiently high cryoprotectant concentrations are achieved within the tissues. To overcome this problem, Towill and Jarret treated the

shoot-tips with 20, 40, 60 and 80% of a solution containing 30% (v/v) glycerol, 15% (v/v) ethylene glycol and 15% (v/v) DMSO during 60, 10, 5, and 10 minutes, respectively, prior to freezing.

Vitrification of biological materials for cryopreservation is, therefore, in theory, a comparatively simple method which was suggested by Luyet in 1937 and used with on nondehydrated mosses, leaves, protozoans, nematodes, erytrocytes, frog muscles and chicken hearts (see review by Armitage and Rich, 1990), and more recently it has again been a focus of strong interest. Bourtron and Mehl (1990), basing their conclusions on various pieces of work, even suggested that vitrification is the only way forward for the future storage of human organs. It now seems that vitrification can also be a good alternative method for plant germplasm conservation as was recently proved for sweet potato (Towill and Jarret, 1992). It is, however, a method which, like others, still presents its own range of problems, yet to be solved; these include the tolerance of cells to high concentrations of cryoprotectant solutions, cooling and thawing methods, stability of glasses to nucleation, cracking at low temperatures and recovery of explants (Armitage and Rich, 1990; Towill and Jarret, 1992).

Chapter 3.1 showed that up to 25% root-tips were able to show FDA activity after they had been slowly frozen to -20°C where they were maintained for 10 minutes, before being plunged into LN (-196°C) for 20

minutes. The technique must, however, be improved, possibly by modifying the cryoprotectant treatment or alternating the cooling rates; another important factor would be the improvement of the method for regenerating the root-tips by inducing them to produce adventitious shoots.

The regeneration of plants from isolated root cultures has been obtained in various species including members of the family Convolvulaceae. (Torrey, 1958; Charlton, 1965; Eapen and Gill, 1986; Rybozynski and Badzian, 1987). The cryopreservation of root-tips could therefore provide an alternative method for germplasm storage in species with shoot meristems that appear to be recalcitrant with regard to the standard procedures.

The production of hairy roots from cultures transformed by genetic engineering is becoming widespread nowadays (Sheng and Yun, 1990; Al-Juboory and Skirvin, 1990; Benson and Hamill, 1991) and cryopreservation proved to be a secure way to store such hairy roots of *Beta vulgaris* and *Nicotiana rustica*, maintaining the biosynthetic capability of the 20% surviving root-tips (Benson and Hamill, 1991).

The regeneration of sweet potato through fibrous root segments is a technique which is recognized as a normal system of propagation (Wilson and Lowe, 1973), producing plants which have the same characteristics as the donors (see review by Peterson, 1975). This fact gives a hope for the maintenance of the genetic integrity of root-tips of sweet potato germplasm stored in LN,

despite the risks of effects of cumulative background radiation (Ashwood-Smith and Friedmann, 1979) and spontaneous mutation which can occur with sweet potato (Clark and Moyer, 1988).

The direct regeneration of plantlets from root cultures was achieved for Jersey Orange and TIB-10 genotypes (see section 4.3). Those results showed similar responses in roots treated with low levels of NAA and in controls. Low auxin concentrations are known to be beneficial to the bud initiation in a few species (Peterson, 1975), however, because of formation of shoot in the controls, it is not clear that the use of NAA was an important factor regarding the shoot formation with the sweet potato root cultures.

Further work is required in this area since the development of a reliable procedure for the direct regeneration of shoots from root cultures would certainly provide an alternative strategy for the cryopreservation of sweet potato germplasm based on the use of root-tips.

The avoidance of the use of growth regulators for the conservation and regeneration of plant germplasm is believed to be one of the most effective ways of maintaining genetic stability (Scowcroft, 1984). New studies must be carried out, preferably using hormone-free methods; the use of high levels of nitrogen could be a good alternative for improving the results reported in this thesis, as this treatment stimulated the production of adventitious buds in detached sweet potato leaves (Wilson, 1973) and also improved the bud initiation on

roots of *Euphorbia esula*, *Bryophyllum proliferum* and *Hieracium florentinum* (see review by Peterson, 1975). The use of a medium supplemented with vitamins and sucrose (see Rybozinski and Badzian, 1987), and changes in the temperature and light regimes (Peterson, 1975) are further factors that have been reported to be effective in promoting the formation of adventitious buds on roots which therefore should be tested with sweet potato root cultures.

Bajaj and Dionne (1966) produced virus X-free *Solanum* root cultures by subculturing the root-tips for three consecutive times every three days. This method was based on the hypothesis that as the root cultures develop quickly they would be more likely to have virus-free apices than shoot-tips. The possibility of producing pathogen-free root cultures could provide a good alternative basis for clonal propagation and germplasm storage.

As suggested in this thesis, root cultures may also provide alternative material for the electrophoretic analysis of esterase isozymes. Esterase isozymes are being used for the characterization of cassava international germplasm collection at CIAT, Colombia (CIAT, 1986), and the fact that each of the seven genotypes tested presented its typical array of bands endorse that esterase zymograms can be a valuable tool for identifying sweet potato genotypes. However, for a more precise identification of the genotype it is better to avoid being restricted to only one enzyme, and the use

of a large number of enzymes is to be recommended (Torres *et al.*, 1978, Withers and Simpson, 1986).

Root cultures are easily obtained from *in vitro* plants and the results indicated that the same cultures could be studied over a period of one month without variation of results. Stegemann (see Huaman and De la Puente, 1988) and Ortega (1987) developed methods of electrophoretic analysis based on the tuberous roots of sweet potato, but the advantage of using root cultures over the methods previously reported are that root cultures are faster and cheaper to produce than tubers and, moreover, the root cultures require reduced space and labour. Nevertheless, those methods developed by either Ortega (1987) or Stegemann (see review by Huaman and De la Puente, 1988) are a good alternatives when *in vitro* root cultures are not readily available.

Isozyme electrophoresis can provide good primary data to estimate genetic diversity, but as it shows limitations in the level of polymorphism and number of loci that are detectable, it is safer to use electrophoresis as complementary to standard characterization using quantitative and qualitative descriptors (Withers and Simpson, 1986; Ramirez *et al*, 1987; Bernatsky and Tanksley, 1989). Nowadays, the use of new molecular techniques such as restriction fragment length polymorphism (RFLPs) (Beckmann and Soller, 1986) and random amplified polymorphic DNA (RAPDs) (Williams *et al.*, 1991; IBPGR, 1993) can provide a very precise means of "finger printing" or characterizing the genomes. RAPD

is based on the use of synthetic 10-mer oligodeoxynucleotides as primers in a DNA-amplification reaction; as only a single primer is used, this amplification does not require previous knowledge of any genomic sequence at the focus being amplified and this is one of the advantages of RAPD over RFLP; other advantages include the use of non-radioactive chemicals and the possibility of automation for high throughput applications (Williams *et al.*, 1991). The use of RAPD is being tested by a group at the University of Birmingham, UK, in collaboration with the International Network for the Improvement of Banana and Plantain (INIBAP) Transit Centre, Leuven, Belgium and IBPGR on the study of somaclonal variation of *Musa* germplasm maintained under slow growth conditions; preliminary results showed a high degree of reproducibility in the identification and discrimination between varieties (IBPGR, 1993). However, the routine use of molecular probes for germplasm characterization and the monitoring of genetic stability is at present limited by cost and by the level of expertise necessary to perform the methods.

The use of embryogenic material for cryopreservation is another alternative, which has not been tested in this programme because of the unreliability of the embryogenic procedures available for sweet potato at the time of the work. Such material from other species has in recent years been tested for cryopreservation, either using the more conventional approaches with cryoprotectants and two stage cooling procedures or embedded in alginate beads

which were dehydrated and frozen without the use of cryoprotectants. Using the first method with oil palm embryoid clumps Elgemann and Dereuddre (1988) obtained up to 80% survival when after a 7 day pre-culture period on 0.75M sucrose containing medium, slow cooling rate (0.5°C.mim^{-1}) was employed prior to freezing. Sudarmonowati (1990) used a two-step cooling procedure with cassava somatic embryos treated with 7% sucrose for 7 days, then 10% (v/v) DMSO prior to freezing, the survival was 91.7% with 25% secondary embryos production. Uragami and colleagues (1993) using unencapsulated somatic embryos of oilseed rape, obtained up to 80-90% survival after a pre-treatment of 60-90 minutes on medium with 1.5 M sucrose followed by partial dehydration in air before the freezing. The alternative approach was followed by Dereuddre and colleagues (1991) with alginate-encapsulated carrot embryos pre-cultured on 0.3 M sucrose-containing medium followed by 4 hours dehydration period prior to freezing; 92% and 71% survival was obtained after direct and two-step freezing, respectively. Uragami and colleagues (1993), obtained up to 95% plantlet recovery with oilseed rape somatic embryos pre-cultured for 1-2 days on 0.75M sucrose prior to encapsulation, dehydration and freezing; Dumet and colleagues (1993), obtained 37-53% survival when oil palm embryos were pre-cultured for 7 days on a medium containing 0.75M sucrose followed by 16 hours dehydration and crypreservation.

The use of dehydrated alginate-encapsulated embryos

for cryopreservation without the use of cryoprotectants, if shown to be widely applicable, could of considerable value and , significantly, somatic embryogenesis has now been successfully achieved with a number of sweet potato genotypes. The most extensive studies have been carried out by Cantliffe and co-workers (Liu and Cantliffe, 1984; Chee and Cantliffe, 1988), although this work has been confined to a single genotype, the cultivar White Star. More recently Al-Mazoorei (personal communication) has achieved success with two further genotypes (Papota and TIB-10) which have been used in the present study. Further work is required, however, to obtain better control of the process with a larger number of genotypes before this approach could be used routinely for cryopreservation of sweet potato germplasm (A. C. Guedes, 1993 personal communication; S. Al-Mazrooai, 1993, personal communication).

Despite the work which has been done towards the collection and conservation of sweet potato germplasm (see review by Huaman and De la Puente, 1988), no definitive method for safe storage has yet emerged. The cryopreservation of root-tips could be a useful alternative to overcome the difficulties presented by some genotypes when freezing techniques are applied to shoot meristems, although the method needs to be improved and tested with a wider range of genetic material. On the other hand, the technique of oil-overlay investigated in this thesis, being cheap and using materials which are readily found in most countries, could be used in any

tissue culture laboratory for the maintenance of active collections.

At the National Centre for Germplasm Resources and Biotechnology (Cenargen/ Embrapa), in Brasilia, Brazil, which is in charge of germplasm conservation in that country; 350 accessions of sweet potato are being stored, using slow growth methods at 20-22°C. At the moment the sweet potato collection is being indexed for viruses and a computerized program is being tested to control all the activities related to the routine of IVAGs; also research is being carried out towards the development of more efficient, secure, cheap and practical routines for the *in vitro* conservation of sweet potato, cassava, yam and potato. The techniques developed in this thesis can be a valuable contribution towards the development of routine procedure for the conservation of the germplasm collections and the research will be continued in Brazil, at Cenargen. New directions, based on the results obtained here and more recently published techniques, will be investigated according to the availability of necessary resources.

Finally it is important to remember that sweet potato produces orthodox seeds and the best way to maintain its germplasm is in seed collections maintained at low temperature (Jones and Dukes, 1982), except for historical clones and material involved in genetic manipulations and secondary product synthesis, which need to be stored *in vitro* if specific genotypes are to be conserved.

* indicates where the reference is to be found in the addendum at the end of this section.

BIBLIOGRAPHY

Acland, J. D.(1971). East African Crops - An introduction to the production of field and plantation crops in Kenya, Tanzania and Uganda. London: Longman.

Al-Juboory, K. and Skirvin, R. M. (1990). Shoot regeneration and petioles of *Agrobacterium* transformed sweet potato (*Ipomoea batatas* Lam.). *Abstracts VIIth. international congress on plant tissue and cell culture*. pp. 86. Amsterdam: International Association of Plant Tissue Culture.

Allan, J. J. (1979). Tissue culture of sweet potato germplasm. Birminghan: University of Birminghan, Ph. D. Thesis.

*

Ashwood-Smith, M. J. and Friedmann, G. B. (1979). Lethal and chromosomal effects of freezing, thawing, storage time, and X-irradiation on mammalian cells preserved at -196^{0}C in dimethyl sulfoxide. *Cryobiology*, **16**, 132-140.

Augereau, J. M.; Courtois, D. and Petiard, V. (1986). Long term storage of callus at low temperature or under mineral oil layer. *Plant Cell Rep.s*, **5**, 372-376.

Austin, D. F. (1977). Hybrid polyploids in *Ipomoea* section *batatas*. *J. Hered.*, **68**, 259-260.

Austin, D. F. (1978). The *Ipomoea batatas* complex. I - Taxonomy. *Bull. Torrey Bot. Club*, **105**, 114-129.

Austin, D. F. (1988). The taxonomy, evolution and genetic diversity of sweet potatoes and related wild species. In: *The exploration, maintenance and utilization of sweet potato genetic resources. Report on the first sweet potato planning conference 1987.* Lima: International Potato Centre.

Bajaj, Y. P. S. (1987). Cryopreservation of potato germplasm. In: *Biotechnology in agriculture and forestry 3. Potato.* Y. P. S. Bajaj (ed.), pp. 472-486. Heidelberg: Springer-Verlag.

Bajaj. Y. P. S. and Dionne, L. A. (1966). Virus X free roots from infected potato plants. *Am. Potato J.*, **43**, 386-394.

Baker, H. G. (1970). Plants and civilization. 2nd. ed. Aylesbury: Hazell Watson & Viley Ltd.

*

Benson, E. E. and Hamill, J. D. (1991). Cryopreservation and post freeze molecular and biosynthetic stability in transformed roots of *Beta vulgaris* and *Nicotiana rustica*. *Plant Cell, Tissue Org. Cult.*, **24**, 163-172.

*

Bonnett, H. T. (1972). Phytochrome regulation of endogenous bud development in root cultures of *Convolvulus arvensis*. *Planta,* **106**, 325-330.

Bonnett, H. T. and Torrey. J. G. (1965). Chemical control of organ formation in root segments of *Convolvulus* cultured *in vitro*. *Plant Physiol.*, **40**, 1228-1236.

*

Bradbury, J. H.; Hammer, B.; Nguyen, T.; Anderson, M. and Millar, J. S. (1985). Protein quantity and quality and trypsin inhibitor content of sweet potato cultivars from the highlands of Papua New Guinea. *J. Agric. Food Chemist.*, **33**, 281-285.

Brewer, G. J. (1970). An introduction to isozyme techniques. New York: Academic Press.

Bridgen, M. P. and Staby, G. L. (1981). Low pressure and low oxygen storage of *Nicotiana tabacum* and *Chrysanthemum* x *morifolium* tissue cultures. *Plant Sci. Letters*, **22**, 177-186.

Broertjes, C. and van Harten, A. M. (1978). Application of mutation breeding methods in the improvement of vegetatively propagated crops. Amsterdam: Elsevier.

Budd, T. W. (1973). An excelent source of vegetative buds for use in plant hormone studies on apical dominance. *Plant Physiol.*, **52**, 82-83.

Buel, C. B. and Weston, W. H. (1947). Applications of mineral oil conservation method to maintaining collection of fungous cultures. *Amer. J. Bot.*, **34**, 555-561.

Carswell, G. K. and Locy, R. D. (1984). Root and shoot initiation by leaf, stem and storage root explants of sweet potato. *Plant Cell, Tissue Org. Cult.*, **3**, 229-236.

Caplin, S. M. (1959). Mineral oil overlay for conservation of plant tissue cultures. *Amer. J. Bot.*, **46**(5), 324-329.

Charlton, W. A. (1965). Bud initiation in excised roots of *Linaria vulgaris*. *Nature*, **207**, 781-782.

*

CIAT. (1986). Annual Report. Biotechnology Research Unit. Cali: International Centre of Tropical Agriculture.

Clark, C. A. and Moyer, J. W. (1988). Compendium of sweet potato diseases. The American Phytopathological Society. Minnesota: APS Press, St. Paul.

Cohen, J. I. (1989). Biotechnology research for the developing world. *TIBTECH*, **7**, 295-303.

Collins, W. W. (1988). Sweet potato in the developing world. In: *Improvement of sweet potato (*Ipomoea batatas*) in East Africa*. Lima: International Potato Centre.

Collins, W. W. and Walter, Jr, W. M. (1982). Potential for increasing nutritional value of sweet potatoes. In: *Sweet potato. Proceedings of the first international symposium* R. L. Villareal and T. D. Griggs (eds.) pp. 355-364. Taiwan: Asian Vegetable Research and Development Centre.

Davis, J. B. and Orstein, L. (1964). Disc electrophoresis-II. Method and application to human serum proteins. *Annals of the New York Academy of Science*, **121**(2), 404-427.

*

Desanero. N. Y. and Rhodes, B. B. (1989). Osmotic stress-induced minimal growth for *in vitro* germplasm storage of sweet potato (*Ipomoea batatas* L.). *Hortscience*, **24**(5), 760.

Dodds, J. H. and Roberts, L. W. (1985). Experiments in plant tissue culture. New York: Cambridge University Press.

*

Eapen, S. and Gill, R. (1986). Regeneration of plants from cultured root explants of mothbean (*Vigna aconitifolia* L. Jacq. Marechal). *Theor. Appl. Genet.*, **72**, 384-387.

Edmond, J.B. and Ammerman, G. R. (1971). Sweet potato: production, processing and marketing. Westport: The AVI Publishers.

*

Esau, K. (1977). Anatomy of seed plants. 2nd. ed. Santa Barbara: John Wiley.

Euroconsult. (1989). Agricultural compendium for rural development in the tropics and subtropics. 3rd. ed. Amsterdam: Elsevier.

FAO. (1990). Production yearbook-1989. Rome: FAO.

Florkowski, W. J. and Jarret, R. L. (1990). The cost alternative strategies for the maintenance of sweet potato germplasm. *Quart. J. Internat. Agric.*, **29**, 79-87.

Frison, E. A. and S. Y. Ng. (1981). Elimination of sweet potato virus disease agents by meristem tip culture. *Trop. Pest Manag.*, **27**(4), 452-454.

Ford-Lloyd, B. and Jackson, M. (1986). Plant genetic resources: an introduction to their conservation and use. London:Edward Arnold.

Frinkle, B. J.; Zavala, M. H. and Ulrich, J. M. (1985). Cryopreservative compounds in the viable freezing of tissues. In: *Cryopreservation of plant cells and organs*. K. Kartha (ed.), pp. 75-113. Boca Raton: CRS Press.

Fouda, S. (1988). Country report on sweet potatoes and future plan in Egypt. In: *Improvement of sweet potato (*Ipomoea batatas*) in East Africa*. Lima: International Potato Centre.

Groth, B. H. (1911). The sweet potato. Contributions from the Botany Laboratory of University of Pennsylvania. University of Pennsylvania.

Grout, B. W. W. and Henshaw, G. G. (1978). Freeze preservation of potato shoot-tip cultures. *Ann. Bot.*, **42**, 1227-1229.

Gunckel, J. E.; Sharp, W. R.; Williams, B. W.; West, W. C. and Drinkwater, W. O. (1972). Root and shoot initiation in sweet potato explants as related to polarity and nutrient media variations. *Bot. Gaz.*, **133**(3), 254-262.

*

Hames, B. D. and Rickwood, D. (1981). Gel electrophoresis of proteins - a technical approach. Oxford: IRL Press.

Henshaw, G. G. (1975). Technical aspects of tissue culture storage for genetic conservation. In: *Crop genetic resources for today and tomorrow*. O.H. Frankel and J. G. Hawkes (eds.) pp. 349-357. Cambridge: Cambridge University Press.

Henshaw, G. G. (1982). Tissue culture methods and germplasm storage. In: *Proceedings of the 5th. international congress of plant tissue and cells culture.* pp. 789-792. International Association of Plant Tissue Culture.

Henshaw, G. G.; O'Hara, J. F. and Stamp, J. A. (1985). Cryopreservation of potato meristems. In: *Cryopreservation of plant cells and organs.* K. Kartha (ed.), pp. 159-170 Boca Raton: CRC Press.

Horton, D. E. (1988). World patters and trends in sweet potato production. *Trop. Agric. (Trinidad),* **65**(3), 268-270.

Horton, D. G.; Prain, G. and Gregory, P. (1989). High level investment returns for global sweet potato research and development. *CIP Circular,* **17**(3), 1-11.

Huaman, Z. (1988). Descriptors for the characterization and evaluation of sweet potato genetic resources. In: *Exploration, maintenance and utilization of sweet potato genetic resources. Report of the first sweet potato planning conference.* Lima: International Potato Centre.

Huaman, Z. and De la Puente, F. (1988). Development of a sweet potato gene bank at CIP. *CIP Circular,* **16**(2), 1-7.

IBPGR. (1983). Advisory committee on *in vitro* storage - report of the first meeting. Rome: Internatinal Board for Plant Genetic Resources.

IBPGR. (1986). Design, planning and operation of *in vitro* genebanks. Rome: Internatinal Board for Plant Genetic Resources,

IBPGR, (1988). Annual report 1987. Rome: Internatinal Board for Plant Genetic Resources.

*

Jarret, R. L. and Florkowski, W. J. (1990). *In vitro* active vs. field genebank maintenance of sweet potato germplasm: major costs and considerations. *Hortsci.*, **25** (2), 141-146.

Jarret, R. L. and Gawel, N. (1991). Abscisic-acid-induced growth inhibition of sweet potato (*Ipomoea batatas* L.) *in vitro*. *Plant Cell, Tissue and Org. Cult.*, **24**, 13-18.

Jones, A. (1967). Should Nishiyama's K133 be designated *Ipomoea batatas*? *Econ. Bot.*, **21**, 163-166.

Jones, A. and Dukes, A. D. (1982). Longevity of stored seed of sweet potato. *Hortsci.*, **17**(5), 756.

Jones, A.; Dukes, P. D. and Schalk, J. M. (1986). Sweet potato breeding. In: *Breeding vegetable crops*. M. J. Basset (ed.) pp. 1-35. The Avi Publishing Co.

Kadir, S. and Rhodes, B. B. (1989). Cryopreservation of *Ipomoea batatas*. *Hortsci.*, **0** (suppl.), 90.

Kahler & Allard, (1970). Genetics of isozyme variants in barley. I- Esterase. *Crop Sci.*, 10, 444-448.

Kartha, K. K. (1981). Meristem culture and cryopreservation - methods and applications. In: *Plant tissue culture - Methods and applications in agriculture*. T. A. Thorpe (ed.) pp. 115-134 Toronto: Academic Press.

Kartha, K. K. (1985). Meristem culture and germplasm preservation. In: *Cryopreservation of plant cells and organs*. K. Kartha (ed.), pp. 115-134. Boca Raton: CRS Press.

Kartha, K. K. (1987). Advances in the cryopreservation technology of plant cells and organs. In: *Plant tissue and cell culture*. T. A. Thorpe (ed.) pp. 447-458 Alan R. Liss, Inc.

Kassan, A. H. (1976). Crops of the West African semi-arid tropics. Hyderabad: ICRISAT.

Latta, R. (1971). Preservation of suspension cultures of plant cells by freezing. *Can. J. Bot.*, **49**, 1253-1254.

Li, H. and Oba, K. (1985). Major soluble proteins of sweet potato roots and changes in proteins after cutting, infection, or storage. *Agric. Biol. Chem.*, **49**(3), 737-744.

*

Lovelock, J. E. and Bishop, M. W. H. (1959). Prevention of freezing damage to living cells by dimetyl sulfoxide. *Nature (London)*, **183**, 1394.

McMillin, D. E. (1983). Plant enzymes: a historical perspective. In: *Isozymes in plant genetics and breeding. part A.* S. D. Tanksley and T. J. Orton (eds.) pp. 3-13. Amsterdam: Elsevier.

Maeshima, M.; Sasaki, T. and Asahi, T. (1985). Characterization of major proteins in sweet potato tuberous roots. *Phytochem.*, **24**, 1899-1902.

Mannonen, L.; Troivonen, L. and Kauppinen, V. (1990). The effect of long term preservation on growth and productivity of *Panax ginseng* and *Catharanthus roseus* cell cultures. In: *VII International congress on plant tissue and cell culture. Abstracts.* pp. 377. Amsterdam: International Association of Plant Tissue Culture.

Martin, F. W. (1982). Analysis of the incompatibility and sterility of sweet potato. In: *Sweet potato. Proceedings of the first international symposium.* R. L. Villareal and

T. D. Griggs (eds.). pp. 275-284. Taiwan: Asian Vegetable Research and Development Centre.

Martin, F. W. (1984). Handbook of tropical food crops. Boca Raton: CRS Press.

Mathur, J.; Mukunthakumar, S.; Grupta, S. N. and Mathur,S. N. (1991). Growth and morphogenesis of plant tissue cultures under mineral oil. *Plant Sci.*, **74**, 249-254.

Mathews, V. H. and Rao, P. S. (1984). *In vitro* production of multiple seedlings from single seeds of mung beans (*Vigna radiata* L. Wilczek). *Z. Planzenphys.*, **113**, 325-329.

Meryman, H. T.; Williams, R. J. and Douglas, M. St. J. (1977). Freezing injury from "solution effects" and its prevention by natural or artificial cryoprotection. *Cryobiology*, **14**, 287-302.

Miranda, J. E. C.; Cruz, C. D. and Pereira, A. S. (1988). Analise de trilha e divergencia genetica de cultivares e clones de batata doce. *Rev. Bras. Genet.*, **11**(4), 881-892.

Moriguchi, T.; Kozaki, I.; Matsuda, N. and Yamaki, S. (1988). Plant regeneration from grape callus stored under a combination of low temperature and silicone treatment. *Plant Cell, Tissue and Org. Cult.*, **11**, 157-161.

Morris, G. J. (1980). Plant cells. In: *Plant cells and low temperature preservation in medicine and biology*. M. J. Ashwood-Smith and J. Farrant (eds.) pp. 253-284. Kent: Pitman Medical.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures *Physiol. Plant.*, **15**, 473-497.

Nakajima, T. and Kawakami, Y. (1969). Root culture and initiation of adventitious bud in cultured root of sweet potato, *Ipomoea batatas* Poiret. *Proc. Crop Sci. Soc. (Japan)*, **38**, 447-458.

Nash, T. (1966). Chemical constitution and physical properties of compounds able to protect living cells against damage due to freezing and thawing. In: *Cryobiology*. H. T. Meryman (ed.). London: Academic Press.

Nishyiama, I. (1971). Evolution and domestication of sweet potato. *Bot. Mag. Tokyo*, **84**, 377-387.

Nishyiama, I. (1982). Evolution of the sweet potato. In: *Sweet potato. Proceedings of the first international symposium*. R. L. Villareal and T. D. Griggs (eds.) pp. 263-274. Taiwan: Asian Vegetable Research and Development Centre.

Nishyiama, I.; Miyazaki, T. and Sakamoto, S. (1975). Evolutionary autoploidy in the sweet potato (*Ipomoea batatas* (L.) Lam.) and its progenitors. *Euphytica*, **24**, 197-208.

Norman, M. J. T.; Pearson, C. J. and Searle, P. G. E. (1984). The ecology of tropical food crops. Cambridge: Cambridge University Press.

O'Brien, P. J. (1972). The sweet potato: its origin and dispersal. *Amer. Anthropolog.*, **74**, 342-365.

Onwueme, I. C. (1978). The tropical tuber crops. Yams, cassava, sweet potato and cocoyams. Chinchester: John Wiley.

Ortega, M. R. V. (1987). Characterization of sweet potato (*Ipomoea batatas* L.) clones by polyacrylamide gel electrophoresis. Bath: University of Bath. MSc thesis.

Peterson, R. L. (1975). The initiation and development of root buds. In: *The development and function of roots*. J. G. Torrey and D. T. Clarkson (eds.). pp.125-161 London: Academic Press.

Polge, C.; Smith, A. U. and Parker A. S. (1949). Revival of spermatozoa after vitrification and dehydration at low temperature. *Nature*, **184**, 666.

Ramirez, H; Hussain, A.; Roca, W. and Bushuk, W. (1987). Isozyme electrophoresis of sixteen enzymes in five tissues of cassava (*Manihot esculenta* Crantz.) varieties. *Euphytica*, **36**(1), 39-49.

Rybozynski, J. J. and Badzian, T. (1987). High regeneration of root segments of *Lotus corniculatus* L. seedlings on hormone-free media. *Plant Sci.*, **51**, 239-244.

Shaw, C. R. and Prasad, R. (1970). Starch gel electrophoresis of enzymes - a compilation of recipes. *Biochem. Genet.*, **4**, 297-320.

Scowcroft, W. R. (1984). Genetic variability in tissue cultures: impact on the germplasm conservation and utilization. Rome: Internatinal Board for Plant Genetic Resources.

Shields, C. R., Orton, T. J.; Stuber, C. W., (1983). An outline of the general resource needs and procedures for the electrophoretic separation of active enzymes form plant tissues. In: *Isozymes in plant genetics and breeding, part A*. S. D. Tanksley and T. J. Orton (eds.). pp. 443-468. Amsterdam: Elsevier.

Sheng, H. S. and Yun, C. S. (1990). Induction and culture of hairy roots of *Glycyrrhiza uralensis* and *G. glabla*. *Abstracts VII international congress on plant tissue and*

cell culture. pp. 76. Amsterdam: International Association of Plant Tissue Culture.

Simpson, B. B. and Orgonzaly, M. (1986). Economic botany: plants in our world. McGraw-Hill.

Simpson, M. J. A. and Withers, L. A. (1986). Characterization of plant genetic resources using isozyme electrophoresis: a guide to the literature. Rome: Internatinal Board for Plant Genetic Resources.

*

Torrey, J. (1958). Endogenous bud and root formation by isolated roots of *Convolvulus* grown *in vitro*. *Plant Physiol.*, **33**, 258-263.

Torrey, J. and Loomis, R. S. (1967). Auxin-cytokiinin control of secondary vascular tissue formation in isolated roots of *Raphanus*. *Amer. J. Bot.*, **54**(9), 1098-1106.

*

Vallejos, C. E. (1983). Enzyme activity staining. In: *Isozymes in plant genetics and breeding, part A*. S. D. Tanksley and T. J. Orton (eds.) pp. 469-515. Amsterdam: Elsevier.

Villareal, R. L.; Lin, S. K.; Chang, L.S. and Lai, S. H. (1979). Use of sweet potato (*Ipomoea batatas*) leaf tips as vegetable. I. Evaluation of morphological traits. *Experiment. Agric.*, **15**, 113-116.

Widholm, J.M. (1972). The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Technol.*, **47**, 189-194.

Williams, J. T. (1989). Practical considerations relevant to effective evaluation. In: *The use of plant genetic resources*. A. H. D. Brown, O. H. Frankel, D. R. Marshall and J. T. Williams (eds.) pp. 235-244. Cambridge: Cambridge University Press.

Williams, J. T. and van Stolen, D. H. (1988). Genetic resources of sweet potato - IBPGR research. In: *Exploration, maintenance, and utilization of sweet potato genetic resources*. Report of the first sweet potato planning conference - 1987. CIP. pp.357-360. Lima: International Potato Centre.

*

Wilson, L. A. (1973). Stimulation of adventitious bud production in detached sweet potato leaves by high levels of nitrogen supply. *Euphytica*, **22**, 324-326.

Wilson, L. A. and Lowe, S. B. (1973). The comparative anatomy of the root system in West Indian sweet potato (*Ipomoea batatas* (L.) LAM) cultivars. *Annals of Bot.* **37**, 633-643.

Wilson, L. A. and Abrams, Jr., C. F. (1982). Mechanization of sweet potato production. In: *Sweet potato. Proceedings of the first international symposium.* R. L. Villareal and T. D. Griggs (eds.) pp. 215-224. Taiwan: Asian Vegetable Research and Development Centre.

Withers, L. A. (1980). Preservation of germplasm. *International review of cytology. Supplement 11B.* B. J. Miflin (ed.), pp. 101-136 Oxford: Oxford University Press.

Withers, L. A. (1984). Germplasm conservation *in vitro*: present state of research and its application. In: *Crop genetic resources: conservation & evaluation.* J. H. Holden and J. T. Williams (eds.) pp. 121-145 George Allem & Unwin. London.

Withers, L. A. (1987). Long-term preservation of plant cells, tissues and organs. In: *Oxford surveys of plant molecular and cell biology.* B. J. Miflin (ed.), pp. 221-272. Oxford: Oxford University Press.

*

Withers, L. A. and Williams, J. T. (1990). Germplasm conservation *in vitro* and cryopreservation. In: *Tecnicas e aplicacoes da cultura de tecidos de plantas.* A. C. Torres and L. S. Caldas (eds.), pp. 267-286. Brasilia: Associacao Brasileira de Cultura de Tecidos de

Plantas/Empresa Brasileira de Pesquisa Agropecuaria - Centro Nacional de Pesquisa em Horticultura.

Yen, D. E. (1970). Sweet potato. In: *Genetic resources in plants*. O. H. Frankel and H. E. Bennett (eds.) pp. 341-350. Oxford: Blackwell

Yen, D. E. (1982). Sweet potato in historical perspective. In: *Sweet potato proceedings of the first international symposium*. R. L. Villareal and T. D. Griggs (eds.) pp. 17-30. Taiwan: Asian Vegetable Research and Development Centre.

ADDENDUM

Armitage, W. J. and Rich, S. J. (1990). Vitrification of organized tissues. *Cryobiology*, 27, 483-491.

Beckmann, J. S. and Soler,M. (1986). Restriction fragment length polymorphisms in plant genetic improvement. *Oxford surveys of plant molecular & cell biology*, 3, 196-250

Bernastsky, R. and Tanksley, S. D. (1989). Restriction fragments as molecular markers for germplasm. In: ***The use of plant genetic resources*** Brown, A. H. D., Frankel, O. H., Marshal, D. R. and Williams,J. T. (eds) pp.353-362.Cambridge: Cambridge University Press.

Bourtron, P. and Mehl, P. (1990). Theoretical prediction of devitrification tendency: determination of critical warming rates without using finite expansions. *Cryobiology*, **27**, 359-377.

Chee, R. P. and Cantliffe, D. J. (1988). Selective enhancement of *Ipomoea batatas* Poir. embryogenic and non-embryogenic callus growth and production of embryos in liquid culture. *Plant cell, tissue and organ culture*, **15**, 149-159.

Dereuddre. J., Blandin, S. and Hassen, N. (1991). Resistance of alginate-coated somatic embryos of carrot (*Daucus carota* L.) to dessication and freezing in liquid nitrogen: 1. Effects of preculture. *Cryo-Lett.*, **12**, 125-134.

Dumet, D., Engelmann, F., Chabrillange, N. and Duval, Y. (1993). Cryopreservation of oil palm (*Elaeis guineensis* Jacq.) somatic embryos envolving a dessication step. *Plant Cell Rep.*, **12**, 352-355.

Engelmann, F. and Dereuddre, J. (1988). Cryopreservation of oil palm somatic embryos: importance of the freezing process. *Cryo-Letters* **9**, 220-235.

Hamon, S, and van Stolen, D. H. (1989). Characterization and evaluation of okra.In: ***The use of plant genetic resources*** Brown, A. H. D., Frankel, O. H., Marshal, D. R. and Williams,J. T. (eds) pp.173-196. Cambridge: Cambridge University Press.

IBPGR, (1993). Annual report 1992 Rome: Internatinal Board for Plant Genetic Resources.

Liu, J. R. and Cantliffe, D. J. (1984). Somatic embryogenesis and plant regeneration in tissue cultures of sweet potato (*Ipomoea batatas* Poir.) *Plan Cell Rep.* 3, 112-115.

Sudarmonowati, E. (1990).Somatic embryogenesis and regeneration in cassava (*Manihot esculenta*Crantz) Bath: University of Bath, Ph.D. Thesis.

Towill, L. E. and Jarret, R. L. (1992). Cryopreservation of sweet potato (*Ipomoea batatas* [L.] Lam.) shoot tips by vitrification. *Plant Cell Rep.* **11**, 175-178.

Uragami, A., Lucas, M.O., Ralambosoa, J., Renard, M. and Dereuddre, J. (1993). Cryopreservation of microspore embryos of oilseed rape (*Brassica napus* L.) by dehydration in air with or without alginate encapsulation. *Cryo-Lett.*, **14**, 83-90.

Williams, J. G. K.; Kubelik, A. R.; Rafalski,J. A. and Tingey, S. V. (1991). Genetic analysis with RAPD markers. In: *More gene manipulations in fungi*. Bennet, J. W. and Lasure, L. L. (eds). pp.431-439. San Diego: Academic Press.

Withers, L. A. (1998). In vitro conservation and germplasm utilisation. In: ***The use of plant genetic resources*** Brown, A. H. D., Frankel, O. H., Marshal, D. R. and Williams,J. T. (eds) pp.309-134. Cambridge: Cambridge University Press.

APPENDIX 1

Murashige and Skoog (1962) basic medium formulation

Ingredients	mgl^{-1}
$CaCl_2.H_2O$	440.00
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FeNa EDTA	36.70
H_3BO_311	6.20
KH_2PO_4	170.00
KI	0.83
KNO_3	1900.00
$MgSO_4.7H_2O$	370.00
$MnSO_4.4H_2O$	22.30
$Na_2MoO_4.2H_2O$	0.25
NH_4NO_3	1650.00
$ZnSO_4.7H_2O$	8.60
Inositol	100.00
Nicotinic acid	0.50
Thiamine HCl	0.10
Pyridoxine HCl	0.50
Glycine	2.00

APPENDIX 2

COMPANIES SUPPLING PRODUCTS USED IN THIS THESIS

Products	Companies
Laboratory chemicals and growth regulators	Sigma Co. Ltd.
Liquid silicone	Dow Corning 200/100cs
Liquid paraffin (heavy)	BDH Co. Ltd.
Agar	Oxoid Co. Ltd
MS basal nutrient medium	Flow Laboratory Co. Ltd.
EtOH and Tween 20	BDH Co. Ltd.
Growth chambers	Satchwell Duotronic
Filtres	Flow Laboratory Co. Ltd.
pH-metre	Orion Research Digital
Balances	Sartorius and Mettler Instruments

APPENDIX 3

Recipe for gel preparation using non-dissociating discontinuous buffer systems

Stock solution	Stack. gel	Resolving gel final acrylamide (%)		
Acrylamide-bisacrylamide (30:0.8)	2.5	12.5	10.0	7.5
Stacking gel buffer	5.0	-	-	-
Resolving gel buffer	-	3.75	3.75	3.75
1.5% ammonium persulfate	-	1.5	1.5	1.5
0.004% riboflavin	2.5	-	-	-
Water	10.0	12.25	14.75	17.25
TEMED	0.015	0.015	0.015	0.015

The first column represent volumes (ml) of the various reagents required to make 15 ml of stacking gel mixture.

The following columns represent volumes (ml) of the various reagents required to make 30 ml of resolving gel mixture.

APPENDIX 4

Davis-Orstein (1964) high pH buffers for non-dissociating discontinuous system

Stacking gel buffer: Tris-HCl (pH 6.8); 6.0 g Tris is dissolved in 40 ml water and is titrated to pH 6.8 with 1M HCl. Water is added to 100 ml final volume.

Resolving gel buffer: Tris-HCl (pH 8.8); 36.3 g Tris and 48.0 ml 1M HCl are mixed brought to 100 ml final volume with water. The solution is titrated to pH 8.8, with HCl, if necessary.

Reservoir buffer: Tris-glycine (pH 8.3) at the correct concentration for use; 3.0 g Tris and 14.4 g glycine are dissolved in and made to 1 litre with water.

APPENDIX 5

RECIPES FOR FIVE ENZYME SYSTEMS TESTED

1)Acid Phosphatase (AcPh) (Li and Oba, 1985)

0.05M acetate buffer pH 5.0

1mM 1-naphthyl phosphate

0.5mg/ml Fast Garnet GBC salt

temperature: $25^{0}C$

2)Esterase (EST) (Kahler and Allard, 1970)

50ml phosphate buffer 0.1M pH 7.0

1ml 1-naphthyl acetate 5mM

1ml 2-naphthyl acetate 5mM

50mg Fast Blue RR salt

temperature: $36^{0}C$

3)Glutamate Oxaloacetic Transaminase (GOT) (Shaw and Prasad, 1970)

100 ml phosphate buffer pH 7.0

73 mg 1-ketoglutaric acid

532 mg L- aspartic acid

200 mg Fast violet B salt

50 mg piridoxal-5-phosphate

temperature: $37^{0}C$

4)Leucine amino peptidase (LAP) (Shaw and Prasad, 1970)

50 ml 0.1M tris-maleate buffer pH 6.0

50 ml H_2O

50 mg Black-K-salt

20 mg L-leucyl-2-naphthylamide

37^0C

5)Peroxidase (PER) (Li and Oba, 1985)

50 ml 0.1M citrate buffer pH 5.0

0.4mg/ml o-phenylenediamine

0.01% (v/v) H_2O_2

temperature: 25^0 C

APPENDIX 6

Rfs VALUES OF ESTERASE ISOZYMES OF 7 TO 37 DAY OLD ROOT CULTURES OF BRONDAL

Age of cultures in days							
7	9	11	13	15	17	19	21
*	0.66	0.66	0.66	*	0.66	0.66	0.67
*	0.70	0.70	0.70	*	0.70	0.70	0.70
*	0.72	0.72	0.72	*	0.71	0.72	0.72
*	0.77	0.77	0.77	*	0.77	0.77	0.77

Age of cultures in days							
23	25	27	29	31	33	35	37
*	0.67	0.66	0.66	*	0.66	0.66	0.66
*	0.70	0.70	0.70	*	0.70	0.70	0.70
*	0.72	0.72	0.72	*	0.72	0.72	0.72
*	0.76	0.76	0.77	*	0.74	0.74	0.74

* missing values

APPENDIX 7

Rfs VALUES OF ESTERASE ISOZYMES OF 7 TO 37 DAY OLD ROOT CULTURES OF CN-1367-2

Age of cultures in days							
7	9	11	13	15	17	19	21
0.63	0.63	0.63	0.64	0.64	*	*	0.64
0.67	0.67	0.67	0.67	0.67	*	*	0.67
0.70	0.70	0.70	0.70	0.70	*	*	0.70
0.73	0.73	0.73	0.73	0.72	*	*	0.72
0.78	0.78	0.78	0.78	0.77	*	*	0.77

Age of cultures in days							
23	25	27	29	31	33	35	37
0.63	*	*	0.63	0.63	*	*	0.63
0.66	*	*	0.66	0.66	*	*	0.66
0.70	*	*	0.70	0.70	*	*	0.69
0.73	*	*	0.73	0.73	*	*	0.73
0.78	*	*	0.78	0.78	*	*	0.78

* missing values

APPENDIX 8

Rfs VALUES OF ESTERASE ISOZYMES OF 7 TO 37 DAY OLD ROOT CULTURES OF JERSEY ORANGE

Age of cultures in days							
7	9	11	13	15	17	19	21
0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.66
0.69	0.69	0.70	0.70	0.69	0.69	0.69	0.70
0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75

Age of cultures in days							
23	25	27	29	31	33	35	37
0.67	0.66	0.66	0.67	*	0.66	0.66	*
0.69	0.69	0.69	0.70	*	0.69	0.69	*
0.74	0.75	0.75	0.75	*	0.74	0.74	*

* missing values

APPENDIX 9

Rfs VALUES OF ESTERASE ISOZYMES OF 7 TO 37 DAY OLD ROOT CULTURES OF TIB-9

Age of cultures in days							
7	9	11	13	15	17	19	21
0.66	0.66	0.67	0.67	0.66	0.66	0.66	0.67
0.69	0.71	0.70	0.71	0.69	0.69	0.69	0.70
0.72	0.74	0.73	0.74	0.72	0.73	0.72	0.73

Age of cultures in days							
23	25	27	29	31	33	35	37
0.65	0.66	0.65	0.66	*	0.66	0.65	0.65
0.69	0.69	0.68	0.70	*	0.68	0.67	0.68
0.71	0.72	0.71	0.71	*	0.71	0.71	0.71

* missing values

APPENDIX 10

Rfs VALUES OF ESTERASE ISOZYMES OF 7 TO 37 DAY OLD ROOT CULTURES OF TIB-10

Age of cultures in days							
7	9	11	13	15	17	19	21
0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67
0.68	0.68	0.69	0.69	0.68	0.68	0.69	0.69
0.74	0.74	0.74	0.74	0.73	0,73	0.73	0.73
0.77	0.77	0.77	0.76	0.76	0.76	0,76	0.76

Age of cultures in days							
23	25	27	29	31	33	35	37
0.67	*	0.67	0.66	0.66	0.66	0.67	0.67
0.68	*	0.69	0.68	0.68	0.68	0.69	0.69
0.72	*	0.73	0.72	0.72	0.72	0.72	0.72
0.75	*	0.76	0.75	0.74	0.74	0.74	0.74

* missing values

APPENDIX 11

Rfs VALUES OF ESTERASE ISOZYMES OF 7 TO 37 DAY OLD ROOT CULTURES OF PAPOTA

Age of cultures in days							
7	9	11	13	15	17	19	21
0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67
0.70	0.70	0.71	0.71	0.71	0.71	0.71	0.71
0.72	0.72	0.73	0.73	0.72	0.72	0.73	0.72
0.74	0.74	0.75	0.75	0.75	0.75	0.75	0.75

Age of cultures in days							
23	25	27	29	31	33	35	37
0.67	0.66	0.66	0.66	0.67	0.66	0.66	0.66
0.71	0.70	0.70	0.70	0.70	0.70	0.70	0.70
0.72	0.72	0.72	0.72	0.73	0.73	0.73	0.73
0.75	0.74	0.74	0.74	0.76	0.76	0.76	0.76

* missing values

APPENDIX 12

Rfs VALUES OF ESTERASE ISOZYMES OF 7 TO 37 DAY OLD ROOT CULTURES OF ROSE CENTENNIAL

Age of cultures in days							
7	9	11	13	15	17	19	21
0.62	0.63	0.63	0.62	0.63	0.63	0.63	0.63
0.67	0.66	0.66	0.65	0.66	0.66	0.66	0.66
0.71	0.70	0.70	0.69	0.70	0.70	0.70	0.70
0.74	0.73	0.73	0.73	0.74	0.73	0.73	0.74
0.78	0.77	0.77	0.77	0.77	0.77	0.77	0.77

Age of cultures in days							
23	25	27	29	31	33	35	37
0.63	0.63	0.62	0.62	0.63	0.63	0.63	*
0.67	0.66	0.66	0.65	0.66	0.66	0.66	*
0.70	0.70	0.70	0.69	0.70	0.69	0.70	*
0.73	0.73	0.73	0.73	0.73	0.73	0.73	*
0.78	0.77	0.77	0.76	0.76	0.76	0.76	*

* missing values